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RESEARCH PAPER

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ORGANIC FOLIAR FERTILISERS CONTAINING CALCIUM, PHOSPHORUS, AND PLANT EXTRACTS FOR THE POTENTIAL CONTROL OF SOME INSECT PESTS OF *Brassica oleracea* var. *capitata* L.

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ABSTRACT

The cabbage whitefly (*Aleyrodes proletella* L.; Hemiptera: Aleyrodidae), the diamondback moth (*Plutella xylostella* L.; Lepidoptera: Noctuidae), and the cabbage aphid (*Brevicoryne brassicae* L.; Hemiptera: Aphididae) are responsible for the most significant losses in cabbage (*Brassica oleracea* var. *capitata* L.) cultivation. In this study, two commercial foliar fertilisers, Mitemine® (a calcium fertiliser) and D-Fense (a phosphorus and potassium fertiliser), and the insecticide Movento 100 SC (spirotetramat) were used to control these pests. In 2020, all treatments applied 6 times reduced pest infestations by approximately 60%–80% compared with the untreated controls. In 2021, a decrease in the number of treatments to 4 resulted in a 10%–20% reduction in effectiveness. Light and scanning electron microscopy revealed variations in the number and density of stomata, cuticle thickness, and leaf structure between the control and treatment groups. Anatomical evaluation suggested that the thickening of the cuticle and epidermis on the abaxial side of the leaves, including the vascular bundles – likely due to the calcium in Mitemine® – may be one of the mechanisms responsible for the observed decrease in the pest population density. The combination of D-Fense and Mitemine® did not significantly alter the effectiveness of Mitemine® against herbivores; however, this combination resulted in a more compact structure of the mesophyll, thicker abaxial epidermis inner cell walls, and a thick layer of cuticle on stomata surface. The findings indicate that foliar fertilisers containing calcium can enhance plant resistance to pests, offering a potential alternative to chemical pesticides in sustainable crop protection strategies.

Keywords: white cabbage, *Brevicoryne brassicae* L., *Aleyrodes proletella* L., *Plutella xylostella* L., mechanical barrier, sustainable crop protection

INTRODUCTION

Cabbage (*Brassica oleracea* var. *capitata* L.) is a globally significant food crop, with Poland ranking 13th in worldwide production [AtlasBig 2018–2025]. This cruciferous vegetable is an important part of many cuisines as well as a valuable source of nutrients, including vitamins C and K, fibre, and various antioxidants [Šamec et al. 2017]. However, Polish cabbage farms face significant challenges from climate changes, particularly rising temperatures, that promote fungal diseases and pest infestations, which

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can severely impact crop yields and quality [Hultgren et al. 2025]. The most economically important insect pests of white cabbage across the world are the cabbage whitefly (*Aleyrodes proletella* L., Hemiptera: Aleyrodidae) [Koca and Kütük 2020, Müller et all. 2024], the diamondback moth (*Plutella xylostella* L., Lepidoptera: Plutelllidae) [Wainwright et al. 2020] and the cabbage aphid (*Brevicoryne brassicae* L., Hemiptera: Aphididae) [Bisht and Kumar 2023]. These insect pests infest cabbages at different growth stages, causing a deterioration in crop quality and a reduction in yield. The cabbage whitefly can cause direct damage through feeding and indirect damage by promoting the growth of sooty mould on leaves [Łabanowski 2015, Hol and Kovaříková 2024]. The diamondback moth, particularly in its larval stage, can cause extensive foliar damage [Gautam et al. 2018], while the cabbage aphid forms dense colonies that can stunt plant growth and transmit viral diseases [Pal and Singh 2013]. Traditionally, these pests have been controlled primarily through the application of insecticides, but their widespread use has led to concerns about environmental impacts, human health risks, and the development of pesticide resistance in target species. Moreover, global trends and European Union directives – Directive (EU) 2023/959 of the European Parliament and of the Council of May 10, 2023 – are pushing for a significant reduction in chemical pesticide use, necessitating the exploration of alternative pest management strategies. In response to these challenges, researchers are investigating various approaches to reduce pest pressure while minimising chemical pesticide use [Shang et al. 2024]. One approach is to seek alternatives to traditional pesticides that work by restricting insect movement or respiration, or by causing death through drying and dehydration [Nile et al. 2019, Susurluk and Bütüner 2024]. Biological agents based on microorganisms, such as entomopathogenic fungi, entomopathogenic nematodes, the bacterium *Bacillus thuringiensis*, and viruses, are also used to control pest populations [Ramanujam 2019, Aynalem et al. 2022, Dede et al. 2022, Bütüner et al. 2024, Chaudhary et al. 2024, Yaraşır et al. 2024]. Another area of research focuses on substances that influence pest growth and development hormonally [Sarvar and Shad 2021]. The use of pheromones, repellents, and attractants is an important aspect of pest management strategies [Larsson 2016, Abd El-Ghany 2019].

There is also a growing focus on enhancing plant resistance to pathogens and pests through both structural and biochemical defences. This approach aims to make the plants themselves less susceptible to pest attacks, reducing the need for external interventions. Structural defences may include thicker cell walls and waxy cuticles that physically impede pest feeding or movement and improve the overall plant toughness and architecture [Seki 2016, Arora and Sandhu 2017, Marasek-Ciolakowska et al. 2021, Biswas et al. 2025]. Biochemical traits, such as odour and taste, may also contribute to insect non-preference [Sandhu et al. 2017]. Numerous studies have shown that the application of macro- and microelements can contribute to increase crop quality and yield, and to improve plant resistance or tolerance to insects [Bala et al. 2018, Tomić et al. 2020, Görlach and Mühling 2021]. Compounds that show promise in this regard include calcium, silicon, and phosphorus. Calcium strengthens plant tissue structures and plays a fundamental role in signalling and supporting plant defence responses [Thor 2019, Sarfraz et al. 2024, Singh et al. 2024, Wdowiak et al. 2024]. Similarly to calcium, silicon exerts physical effects on pests by accumulating in epidermal cells, reducing pest adaptability due to decreased digestibility and feeding preferences [Kvedaras et al. 2007, Massey and Hartley 2009, Strömberg et al. 2016, Frew et al. 2019]. Additionally, silicon plays a critical role in inducing plant biochemical defence mechanisms against pests [Gomes et al. 2005, Rahman et al. 2015]. Phosphorus is also vital because it enhances plant regeneration, root and shoot growth, collectively improving general resistance to insects [Bala et al. 2018].

The hypothesis for the present study is that mineral fertilisers strengthen cell walls, creating effective barriers against sap-sucking insects. This hypothesis is based on the understanding that nutrient management can significantly influence plant physiology and, consequently, pest interactions. Mineral fertilisers, which combine organic matter with essential minerals, may provide a balanced nutrient profile that supports both plant growth and defence mechanisms. To test this hypothesis, we assessed the effects of mineral fertilisers, applied alone or in combination, on pest population dynamics and changes in anatomical characteristics of

leaves of *B. oleracea* var. *capitata* L. that may contribute to enhanced pest resistance. We applied two commercial foliar fertilisers – Mitemine® (a calcium fertiliser) and D-Fense (a phosphorus and potassium fertiliser) containing organic extracts of *Allium sativum* L., *Sesamum indicum* L., and *Salix alba* L. – to control the population of cabbage whitefly, cabbage aphid, and diamondback moth. Moreover, we discuss the possible increase in mechanical defences in white cabbage after foliar fertiliser application. By evaluating the efficacy of mineral fertilisers in pest management, this research contributes to the development of more sustainable agricultural practices. If successful, this approach could offer cabbage farmers an environmentally friendly tool to reduce pest damage while potentially improving crop quality and yield.

MATERIALS AND METHODS

Plant materials

White cabbage 'Ditmarska' plants were planted in sandy loam soil (soil quality class IVa, soil pH 5.7–6.0, and 1.7% organic matter) on 21 April 2020 and 17 May 2021, in the experimental field of the National Institute of Horticultural Research in Skierniewice, Poland (51°57'50.6"N, 20°10'15.2"E). The experiment employed a randomised block design with five replicates, with each plot comprising 40 plants (10 m^2 per plot). The plants were irrigated via a sprinkler system, as required during the experiments, with 20 mm cm^{-2} applied per irrigation event. Fertilisation consisted of phosphorus and potassium (superphosphate, 130 kg ha^{-1}; and potassium sulphate 160 kg ha^{-1}) applied pre-sowing. Nitrogen was applied pre-sowing (nitro-chalk, 130 kg ha^{-1}) and post-sowing (nitro-chalk, 70 kg ha^{-1}).

Experimental design

Four treatments were applied in 2020 and 2021 multiple times throughout the growing season, as detailed in Table 1. The control group (1) received no foliar fertiliser or pesticide treatment. The Mitemine® (Cosmocel SA) group (2) received this foliar calcium fertiliser (10% Ca and 14% CaO) along with plant extracts (2.5% *A. sativum* and 1.25% *S. indicum*). The group (3) received Mitemine® (1.5 L ha^{-1}) and D-Fense (Cosmocel S.A., 1 kg ha^{-1}) alongside the adjuvant Inex-A (50 ml 100 L^{-1} water). D-Fense is a phosphorus and potassium foliar fertiliser (31% P_2O_5 and 52% K_2O) that contains 4% of an organic extract from *S. alba* (at concentration of 1%) and 10,000 ppm phenolic acid. Inex-A is a non-ionic surfactant with wetting and penetrating action; it improves the distribution and spreading of substances on the surface and in the tissues of plants. The group (4) received Movento® 100 SC (Bayer) (100 g L^{-1} of spirotetramat, an insecticide) applied at 0.75 kg ha^{-1}.

Table 1. Description of the experimental treatments in 2020 and 2021

	Treatment	Dose	The number and dates of applications	
1	Control (no foliar fertilisers or pesticide treatment)	–	2020	2021
2	Mitemine®	1.5 L ha^{-1}	Six applications: 11, 18, and 25 May; 15, 22, and 29 June	Four applications: 12, 18, and 25 June; 2 July
	Inex-A	50 mL per 100 L of solution		
3	Mitemine®	1.5 L ha^{-1}	Six applications: 11, 18, and 25 May; 15, 22, and 29 June	Four applications: 12, 18, and 25 June; 2 July
	Inex-A	50 mL per 100 L of solution		
	D-Fense	1 kg ha^{-1}		
4	Movento 100 SC	0.75 L ha^{-1}	Two applications: 11 May; 15 June	Two applications: 12 and 25 June

Pest population assessment

Pest population assessments in both 2020 and 2021 were conducted on 25 randomly selected cabbage plants in each plot. The number of individuals of each species was recorded as they appeared on the same plants over time. The first observation was made when the plants were at the 9-leaf stage (BBCH 19), while the last observation was made when the heads reached 80% of the typical size (BBCH 48).

For the *A. proletella* the number of adults, egg batches, and larvae were counted. The counting of adult whiteflies was conducted early in the day, when temperatures were lower and the adults were less active. The first assessment was performed immediately before application, and the subsequent assessments were performed every 7 days, that is, on each application date. For *P. xylostella* the number of live caterpillars of different ages on all 25 plants was counted. The first assessment was performed immediately before application, while the subsequent assessments were performed every 7 days that is, on each application date. For the *B. brassicae* the number of living aphids on all 25 plants was counted. For both years, the first assessment was performed immediately before the application. In 2020, the second observation was made 3 days after the first application, whereas in 2021, it was made 7 days after the first treatment. The subsequent assessments were performed every 7 days, that is, each date of application.

The influence of the treatments on histological structure of leaves

Fourteen days after the last treatment, white cabbage plants were collected to assess the effects of foliar fertiliser on the anatomical traits of the leaves. To observe stomata with a light microscope (Eclipse 80i, Nikon, Tokyo, Japan), the abaxial epidermis was isolated from the third leaf using adhesive tape, 10 cm from their apices, and stained with 2% toluidine blue according to the method described by Dyki and Habdas [1996]. For each treatment, the stomatal density per square millimetre (n = 5 replications) and the stomatal length (n = 3 replications × 100 stomata) were determined. To observe the leaf anatomy, 10 mm × 5 mm pieces of the third leaf were cut for each treatment. The material was fixed with the CrAF (1% chromic acid, 1% acetic acid, and 50% formalin) solution for 48 h at room temperature, dehydrated using an ascending alcohol series (70%, 80%, 90%, and 100%), and embedded in paraffin according to a previously reported method [Marasek-Ciolakowska et al. 2020]. Transverse sections, 12 μm thick, were cut with a rotary microtome (Leica, Wetzlar, Germany) and stained with 1% safranin and 0.5% fast green. The sections were mounted in Canada balsam and analysed with the same light microscope that was used to observe stomata. For each leaf sample, the thickness of the lamina, the abaxial epidermal layer on the nerves and between the nerves, and the cuticle on the nerves and between the nerves was determined. For statistical analysis, seven replicates were used for each treatment, and each replicate consisted of 20 measurements. The surface and anatomy of leaf was examined using an Eclipse 80i microscope with the NIS-Elements BR ver. 2.30 program (Nikon, Tokyo, Japan) at 100× and 400× magnification.

Scanning electron microscopy was used to examine the leaf ultrastructure. Fragments of the third leaf (10 mm × 5 mm) were fixed with the CrAF solution, dehydrated in an ascending alcohol series (70%, 80%, 90%, and 100%), desiccated using critical point drying with CO_2, and sputter-coated with gold [Pathan et al. 2008]. There were three replicates for each treatment. The micromorphology of the leaf surface and the internal structure of the leaf was analysed using a JSM 6390LV scanning electron microscope (JEOL, Japan) at the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw.

Statistical methods

To analyse comprehensively the degree of plant infestation by pests throughout the experiment, the cumulative insect-day (CID) index was determined for each year [Ruppel 1983].

$$CID = \Sigma\ 0.5 \times (P_a + P_b) \times D_{a\text{-}b},$$

where P_a and P_b are the average number of insects per leaf on two successive sampling dates, and $D_{a\text{-}b}$ is the number of days between the two sampling dates.

The influence of various preparations on the histological structure of leaves and insect populations were analysed using one- or two-way analysis of variance (ANOVA; foliar fertiliser or pesticide treatment ×

study year). To stabilise variance, when necessary, the data were subjected to logarithmic transformation. The Newman–Keuls test was used to determine differences between the groups ($\alpha = 0.05$). Statistical analysis was performed with the STATISTICA v.13 program (StatSoft, Tulsa, OK, USA).

The efficacy of the applied treatments against pests was calculated using Abbott's formula:

$$\text{Efficacy (\%)} = \frac{\text{Infestation in control group} - \text{Infestation in treated group}}{\text{Infestation in control group}} \times 100.$$

RESULTS

The influence of the treatments on the pest populations

In both years of the study, the applied treatments reduced the population sizes of the studied insect species (Figs. 1–4) The efficacy of the treatments was similar in 2020 and 2021, with most exceeding 60% and, in some cases, reaching over 80% (Tab. 2).

In most cases, the two-factor ANOVA (treatment × year) did not reveal any statistically significant differences in the effectiveness of the tested protection programs in reducing the populations of the analyzed pest species. The only exception was observed for cabbage whitefly larvae, where the effectiveness of Movento 100 SC in 2021 was significantly higher compared to the combination of Mitemine® + Inex-A + D-Fens used in 2020. For the 'treatment' factor, the following F and p values were obtained: cabbage whitefly – adults: $F_{2,24} = 0.845$, $p = 0.442$; eggs: $F_{2,24} = 0.363$, $p = 0.700$; larvae: $F_{2,24} = 3.891$, $p = 0.034$; diamondback moth caterpillars: $F_{2,24} = 1.248$, $p = 0.305$; and cabbage aphids: $F_{2,24} = 0.333$, $p = 0.720$.

Aleyrodes proletella L.

In 2020, the first cabbage whitefly adults and eggs were recorded on cabbage on 15 June. The number of adults ranged from 1.9 to 2.7 individuals per plant (Fig. 1A), while the number of eggs ranged from 1.4 to 1.8 individuals per plant (Fig. 1C). Cabbage whitefly larvae were found both on the control plants and the plants treated with Mitemine® + Inex-A + D-Fense groups as late as 22 June, but their abundance did not exceed 1 individual per plant (Fig. 1E). In 2021, the first cabbage whitefly adults were observed on 12 June, and their abundance did not exceed 1 individual per plant (Fig. 1B). Besides the control plants, there was a small number of eggs found for the plants treated with Mitemine® + Inex-A + D-Fense (Fig. 1D). Larvae did not appear until 2 weeks later, that is, 24 June, at approximately 2 individuals per plant for the control plants and < 1 individual per plant for the treated plants (Fig. 1F).

Based on the two-way ANOVA (treatment × year), the treatment had a significant effect on the level of infestation by the cabbage whitefly, as measured with the CID. All the applied treatments similarly reduced the abundance of the cabbage whitefly compared with the control plants. This reduction occurred for the adults ($F_{3,28} = 32.948$, $p < 0.001$), eggs ($F_{3,28} = 18.585$, $p < 0.001$), and larvae ($F_{3,28} = 26.019$, $p < 0.001$) of this species. In addition, the pressure of the pest was significantly higher in 2021 than in 2020, ($F_{1,28} = 6.423$, $p = 0.017$ for adults, $F_{1,28} = 17.749$, $p < 0.001$ for eggs, and $F_{1,28} = 26.223$, $p < 0.0001$ for larvae) (Fig. 4A–C).

Plutella xylostella L.

In 2020, the first caterpillars of the diamondback moth were observed on 15 June, whereas in 2021, they appeared on 12 June. The initial infestation of the plants was higher in 2020, ranging from 2.3 to 4 individuals per plant, whereas in 2021, the number did not exceed 0.38 individuals per plant (Fig. 2). This difference was reflected later in the season, as the level of infestation, measured using the CID, was significantly higher in 2020 than in 2021 ($F_{1,28} = 111.654$, $p < 0.0001$). Over the 2 years of the research, there was a significant reduction in pest pressure, measured with the CID index, in the treated plants compared with the control plants ($F_{3,28} = 34.070$, $p < 0.0001$) (Fig. 4D).

Brevicoryne brassicae L.

In 2020, the first wingless cabbage aphids were observed as early as 11 May. Their abundance was low, ranging from 0.14 to 0.42 individuals per plant (Fig. 3A). Each treatment reduced the aphid population below the level observed in the control plants. However, 21 days after the third treatment, aphid abundance increased for all tested treatments, reaching an abundance ranging from 2.26 individuals per plant for

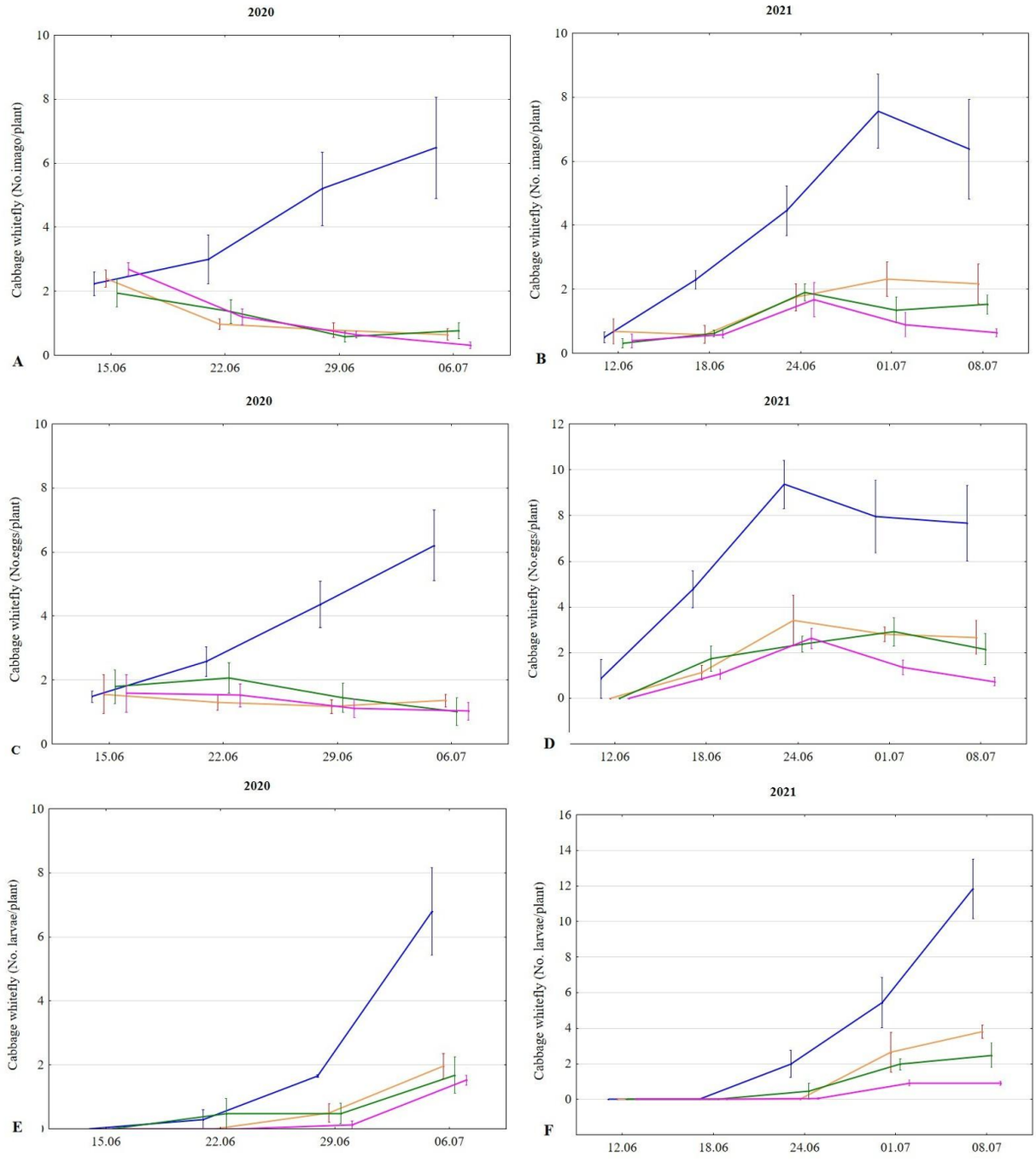

Fig. 1. The effect of the treatments on cabbage plant colonisation by *Aleyrodes proletella* L. (mean ± standard error of the mean) in (A, C, and E) 2020 and (B, D, and F) 2021. The panels show the number of (A and B) adults, (C and D) eggs, and (E and F) larvae. Legend: control; Mitemine® + Inex-A; Mitemine® + Inex-A + D-Fense; Movento100 SC

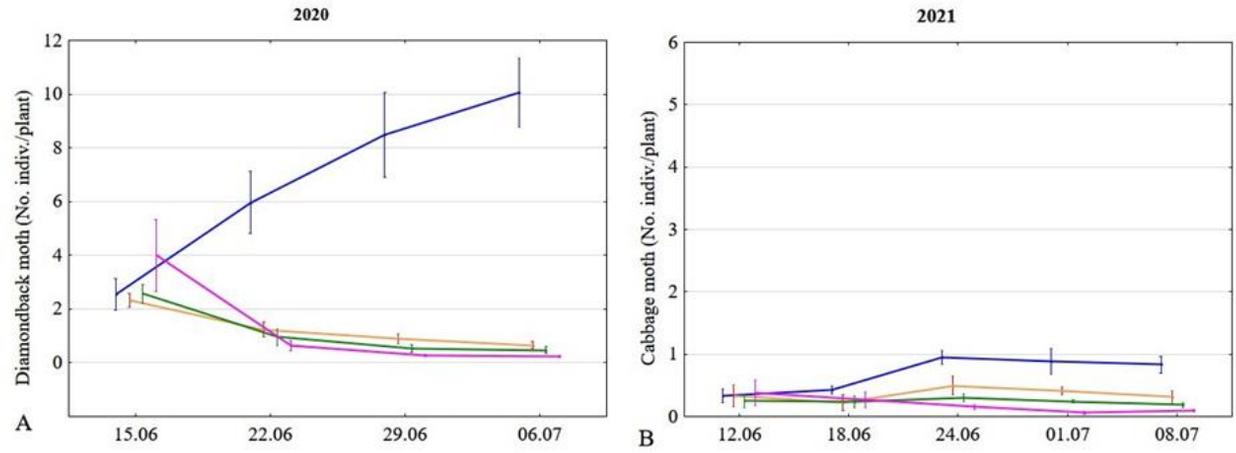

Fig. 2. The effect of the treatments on cabbage plant colonisation by *Plutella xylostella* L. (mean ± standard error of the mean) in (A) 2020 and (B) 2021. Legend: control; Mitemine® + Inex-A; Mitemine® + Inex-A + D-Fense; Movento100 SC

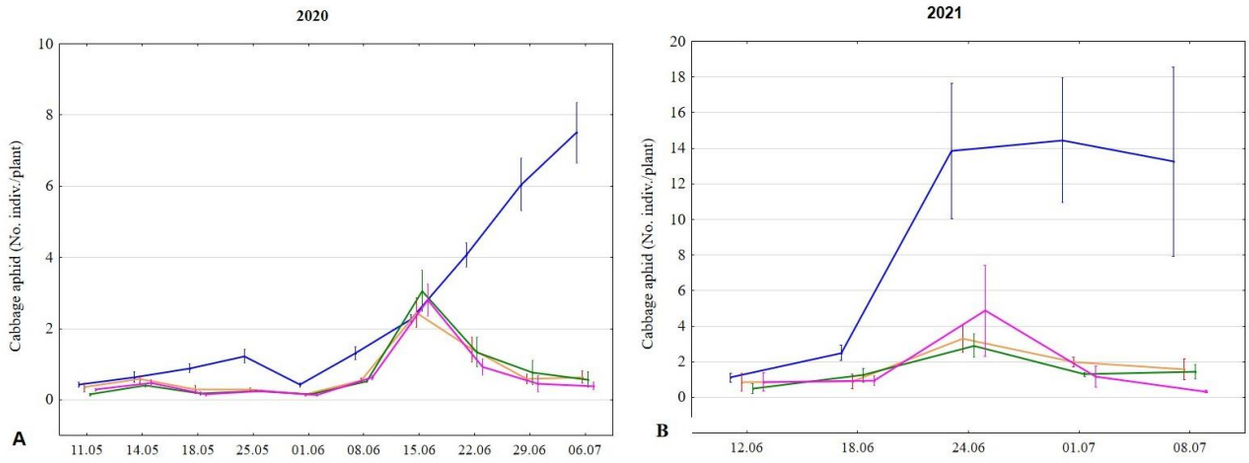

Fig. 3. The effect of the treatments on cabbage plant colonisation by *Brevicoryne brassicae* L. (mean ± standard error of the mean) in (A) 2020 and (B) 2021. Legend: control; Mitemine® + Inex-A; Mitemine® + Inex-A + D-Fense; Movento100 SC

the control plants to 3.06 individuals per plant for the plants treated with Mitemine® + Inex-A + D-Fense. A second spraying with Movento 100 EC, as well as a further application of fertiliser, reduced the abundance of cabbage aphids to < 1 individual per plant, while the abundance of aphids on the control plants increased to > 7 individuals per plant.

In 2021, cabbage aphids were found on plants on 12 June (Fig. 3B). The abundance ranged from 0.50 individuals per plant for the plants treated with

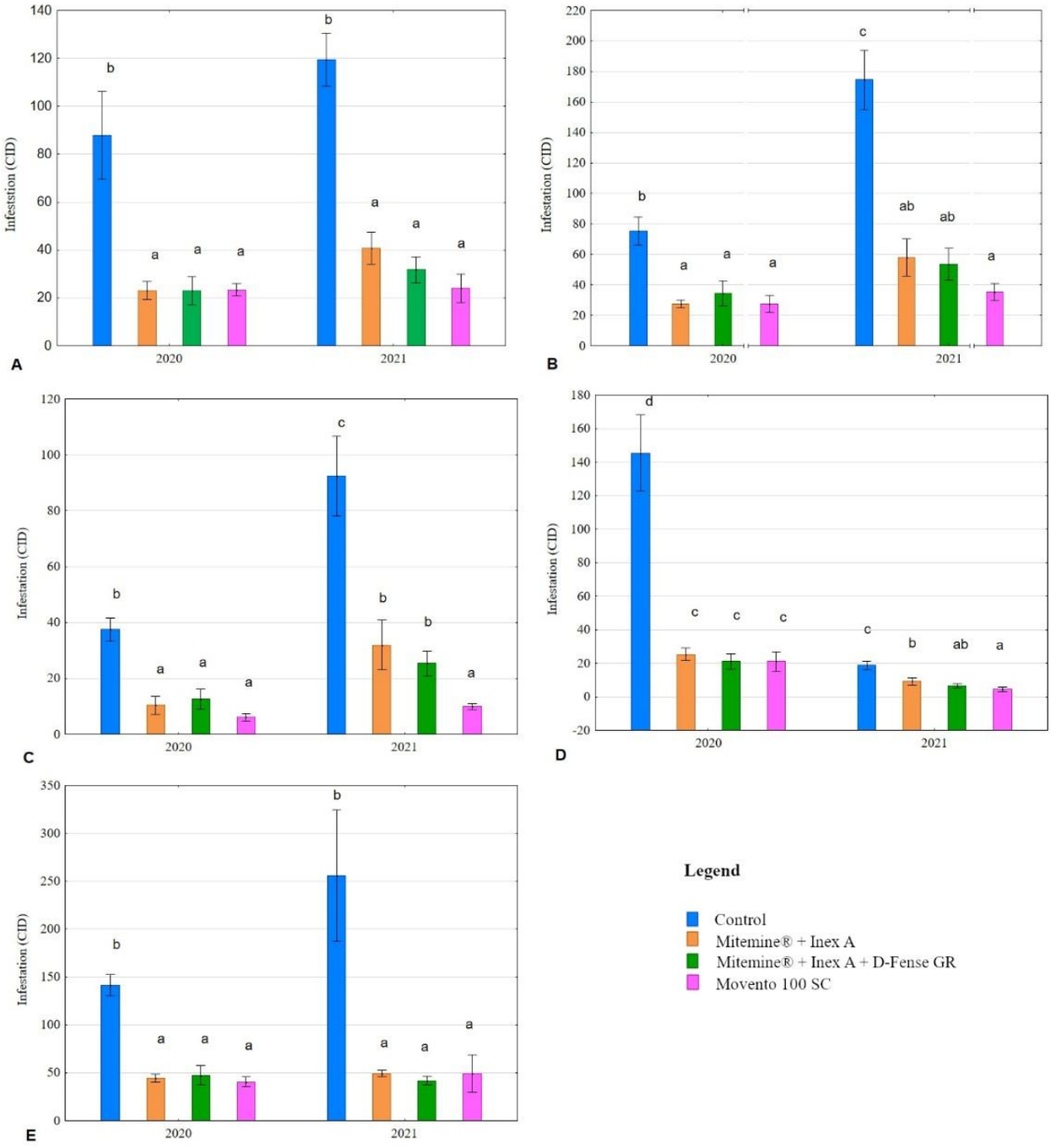

Fig. 4. The influence of the treatments on the mean (± standard error of the mean; n = 12) cumulative insect-days (CID) for cabbage whitefly (A) adults, (B) eggs, and (C) larvae; (D) diamondback moths; and (E) cabbage aphids in 2020 and 2021. For each graph, bars with the same letter do not differ significantly (two-way ANOVA followed by the Newman–Keuls test; $\alpha = 0.05$)

Table 2. Effectiveness of treatments against selected insect species in 2020 and 2021. Within each column, means followed by the same letter do not differ significantly (two-way ANOVA followed by the Newman–Keuls test; α = 0.05)

Treatment	Efficacy of treatments according to Abbott's formula (%)				
	Cabbage whitefly adults	Cabage whitefly egg batches	Cabbage whitefly larvae	Diamondback moth caterpillars	Cabbage aphids
2020					
Mitemine® + Inex-A	72.4 a	62.0 a	70.7 ab	80.4 a	68.1 a
Mitemine® + Inex-A + D-Fens	74.6 a	54.3 a	62.1 a	85.3 a	66.0 a
Movento 100 SC	70.2 a	58.7 a	83.8 ab	83.0 a	70.0 a
2021					
Mitemine® + Inex-A	65.7 a	63.0 a	72.4 ab	57.5 a	76.9 a
Mitemine® + Inex-A + D-Fens	73.9 a	69.0 a	70.7 ab	65.5 a	80.4 a
Movento 100 SC	78.6 a	77.5 a	89.0 b	75.6 a	82.5 a

Mitemine® + Inex-A + D-Fense to 1.12 individuals per plant for the control group. After 5 weeks, the abundance of aphids increased to 13.6 individuals per plant for the control plants, 1.58 individuals per plant for Mitemine® + Inex-A treatment, 1.43 individuals per plant for Mitemine® + Inex-A + D-Fense treatment, and 0.3 individuals per plant for Movento 100 SC treatment.

Two-way ANOVA revealed that the treatments significantly reduced the degree of cabbage aphid infestation measured with the CID index ($F_{3,28}$ = 26.554, $p < 0.0001$). Although the control plants showed higher infestation in 2021 than in 2020, the year effect was not significant ($F_{1,28}$ = 0.657, p = 0.425) (Fig. 4E).

The influence of the treatments on histological structure of the leaves

In the control plants, the difference between palisade and spongy mesophyll was clearly visible in the cross section (Fig. 5A). The cells of the spongy parenchyma were smaller than those of the palisade parenchyma and were loosely arranged. There treatments have variable effects on the anatomical structures of the leaves (Fig. 5B–H). In the leaves treated with Mitemine® + Inex-A or Movento 100 SC, the mesophyll cells had a looser structure and larger cellular spaces (Fig. 5B and D). In the cross-sections of the leaves treated with Mitemine® + Inex-A + D-Fense, the cells of the palisade and spongy mesophyll were tightly arranged, and small intercellular spaces were localised mostly above the stomata (Fig. 5C).

The treatments had a significant effect on the thickness of the cuticle of the lower side of the leaves ($F_{3,24}$ = 3.4188, p = 0.03342). This structure was the thickest for the leaves from the plants treated with Movento 100 SC and the thinnest for the leaves from the control plants. In addition, the leaves from the plants treated with Mitemine® + Inex-A or Mitemine® + Inex-A + D-Fense were thicker than the leaves from the control plants, although the differences were not significant (Fig. 6A).

For the cuticle on the vascular bundles of the lower side of the leaf, the only significant difference was between the leaves from the control plants and the leaves from the plants treated with Mitemine® + Inex-A ($F_{3,24}$ = 3.9594, p = 0.01997) (Fig. 6B). A significant difference in the thickness of the cuticle of the lower side of the leaves ($F_{3,24}$ = 3.9594, p = 0.01997) has been found. Specifically, in the leaves from the control plants, this structure was significantly thinner

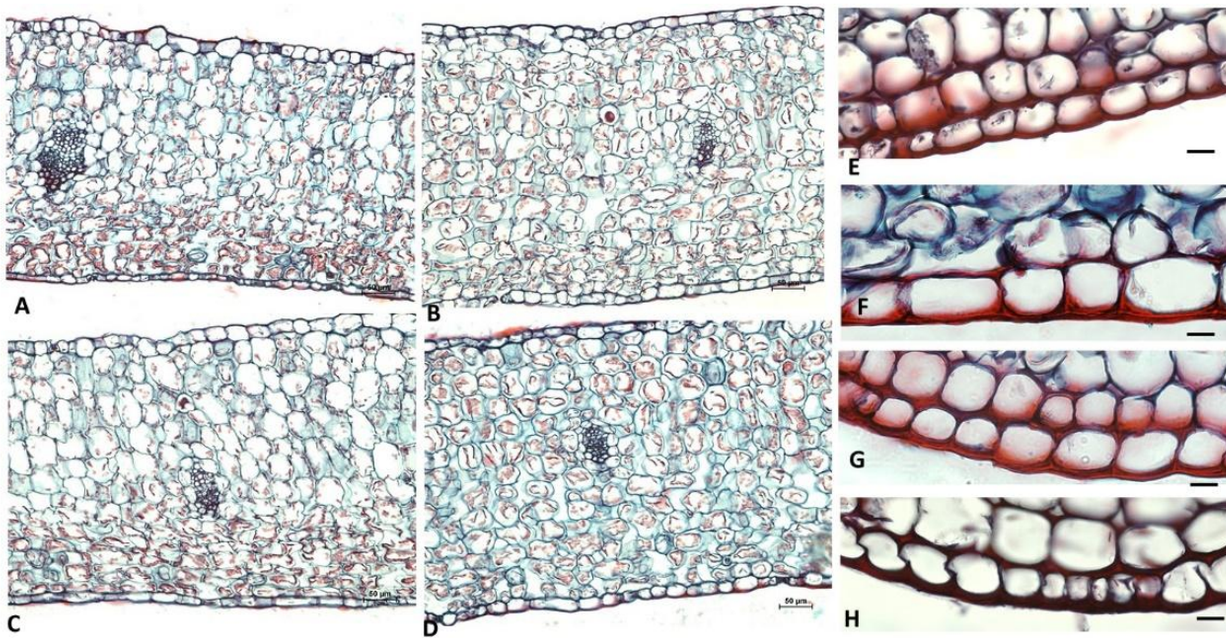

Fig. 5. Anatomical traits of white cabbage leaves stained with safranin-fast green and observed under a light microscope. (A–D) Cross-sections of a leaf blade. (E–H) The size of abaxial epidermis cells at the vascular bundles. The scale bars represent 10 µm. (A and E) Untreated (control); (B and F) Mitemine® + Inex-A; (C and G) Mitemine® + Inex-A + D-Fense; (D and H) Movento 100 SC

compared with the leaves from the plants treated with Mitemine® + Inex-A or Movento 100 SC (Fig. 6C). The tretments had a significant effect on the cuticle of the abaxial side of the leaves measured on the vascular bundles ($F_{3,24}$ = 8.0860, p = 0.00068): the epidermis was thicker for the vascular bundles of the leaves from the plants treated with all tested preparations compared with the leaves from the control plants (Fig. 5E–H and 6D). The veins of the leaves from the plants treated with Mitemine® + Inex-A showed the thickest skin, but it was not significantly different compared with the leaves from the plants treated with Mitemine® + Inex-A + D-Fense or Movento 100 SC.

The treatment also significantly affected the stomatal density ($F_{3,16}$ = 58.595, p = 0.00000) and length ($F_{3,16}$ = 38.694, p = 0.00000). The leaves from the control plants had a significantly higher stomatal density compared with the leaves from the plants that were treated with the tested preparations (Fig. 6E). In contrast, the leaves from the plants treated with Mitemine® + Inex-A + D-Fense or Movento 100 SC presented longer stomata (Fig. 6F).

The anatomy of the lower (abaxial) leaf surface of white cabbage was examined with a light microscope and a scanning electron microscope (Fig. 7). Staining of the isolated abaxial epidermis with toluidine blue (for examination with a light microscope) revealed the presence of a thick cuticle layer on some stomata in the epidermis of the leaves from the plants treated with Mitemine® + Inex-A + D-Fense (Fig. 7C). The scanning electron micrographs of the leaf showed that leaf surface was not flat, but rather consisted of bumps/protrusions (Fig. 7E–H). There were anisocytic, elliptical, and outlined stomata below the epidermal surface (sunken stomata). Similarly to the observations made under the light microscope, there were differences in the surface ultrastructure for the leaves from the plants treated with Mitemine® + Inex-A + D-Fens, where the thick layer of cuticle-covered stomata and protrusions were less distinct (a less folded epidermis) (Fig. 7C). There was no difference in the leaf surface ultrastructure between the control plants and the plants treated with Mitemine® + Inex-A or Movento 100 SC, except for the stomatal size and density.

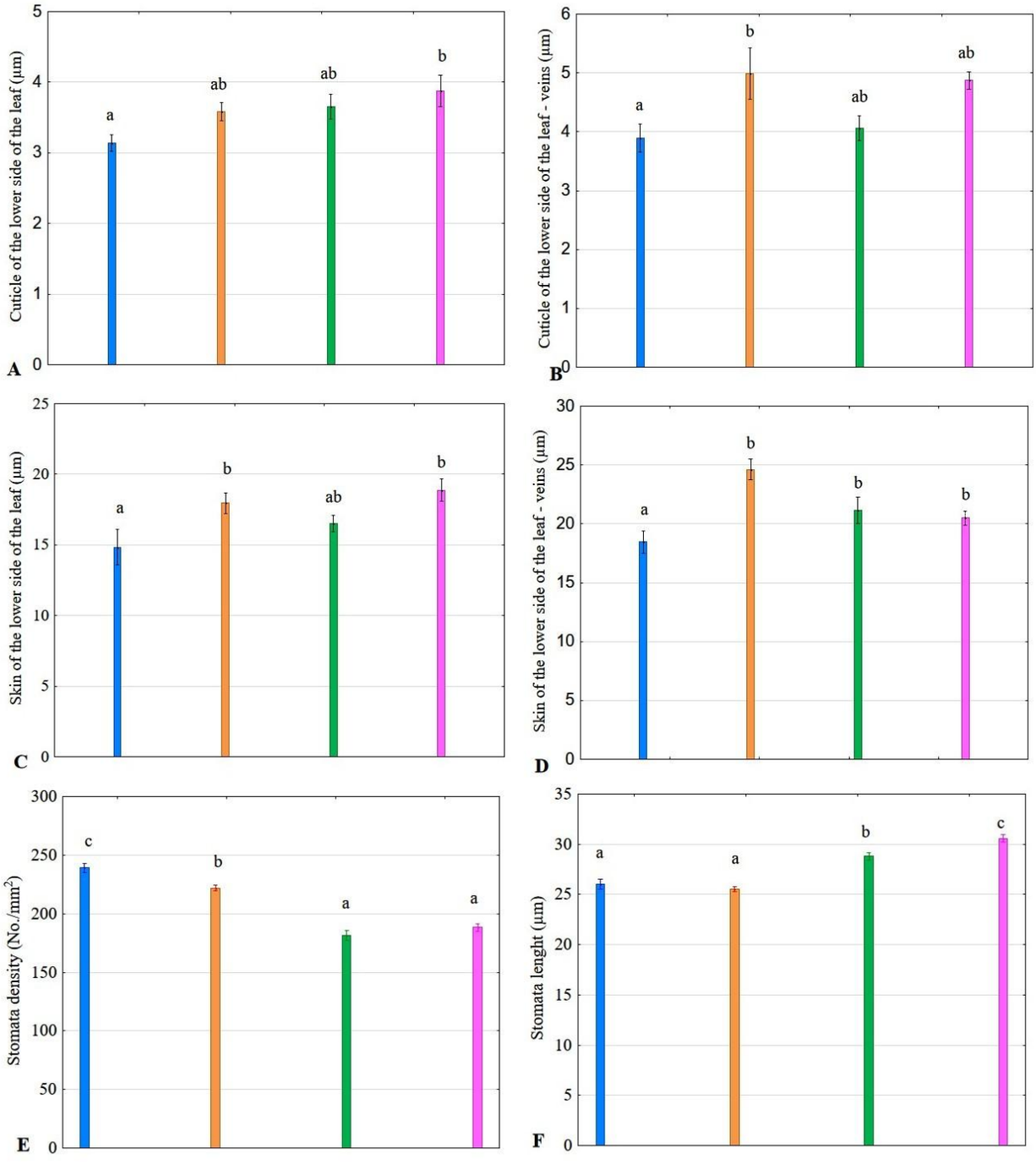

Fig. 6. The influence of the treatments on the histological structures of the lower side of white cabbage leaves. The graphs show (A and B) the cuticle, (C and D) the skin, and (E and F) the stomata. For each graph, the bars with the same letter do not differ significantly (one-way ANOVA followed by the Newman–Keuls test; $\alpha = 0.05$). Legend: Control; Mitemine® + Inex-A; Mitemine® + Inex-A + D-Fens; Movento 100 SC

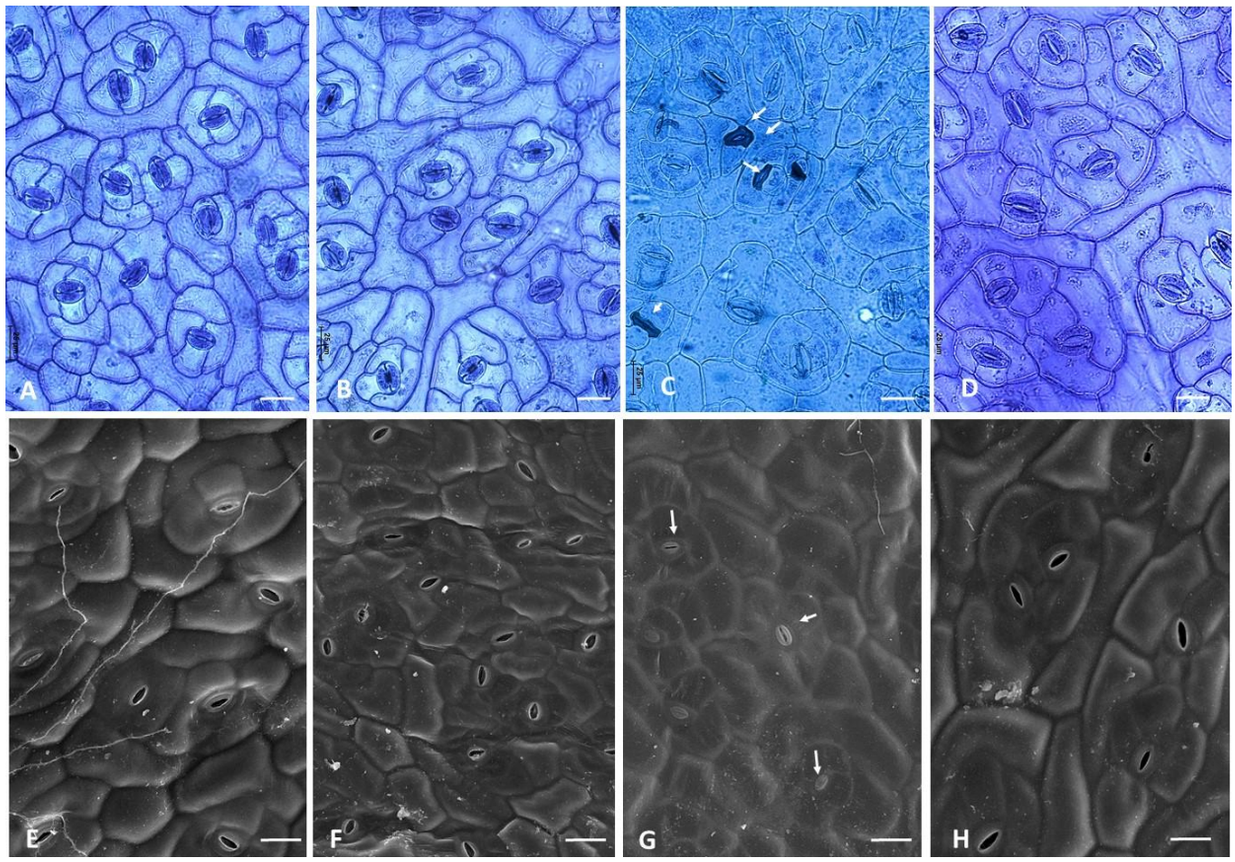

Fig. 7. The influence of the treatments on the abaxial epidermis of white cabbages as observed with (A–D) a light microscope and (E–H) a scanning electron microscope. The images are from (A and E) control plants, (B and F) plants treated with Mitemine® + Inex-A, (C and G) plants treated with Mitemine® + Inex-A + D-Fense, and (D and H) plants treated with Movento 100 SC. The white arrows indicate stomata with thick cuticle layers. The scale bars represent 25 µm

In the leaf cross-sections observed under a scanning electron microscope (Fig. 8), the leaves from the plants treated with Mitemine® + Inex-A + D-Fense presented more turgid and rigid cell walls compared with the leaves from the plants treated with the other preparations. In the leaves from the plants treated with Mitemine® + Inex-A or Movento® 100 SC, the mesophyll cells had a looser structure and larger cellular spaces.

One-way ANOVA revealed significant differences in the epidermal wall thickness between the treatments ($F_{3,36}$ = 59.108, $p < 0.00001$). The leaves from the plants treated with Mitemine® + Inex-A + D-Fense had thicker inner cell walls of the lower epidermis (0.91–1.68 µm) compared with the leaves from the control plants (0.34–0.40 µm) and the plants treated with the other preparations (0.54–1.05 µm) (Fig. 8G). The palisade and spongy mesophyll cells of the leaves from the plants treated with Mitemine® + Inex-A + D-Fense were tightly arranged (Fig. 8C).

DISCUSSION

We have demonstrated that the foliar fertiliser Mitemine®, applied alone or in combination with D-Fense as a spray six or four times during the growing season, could effectively control cabbage whiteflies, diamondback moth caterpillars, and cabbage aphids. Its effectiveness was comparable to the ref-

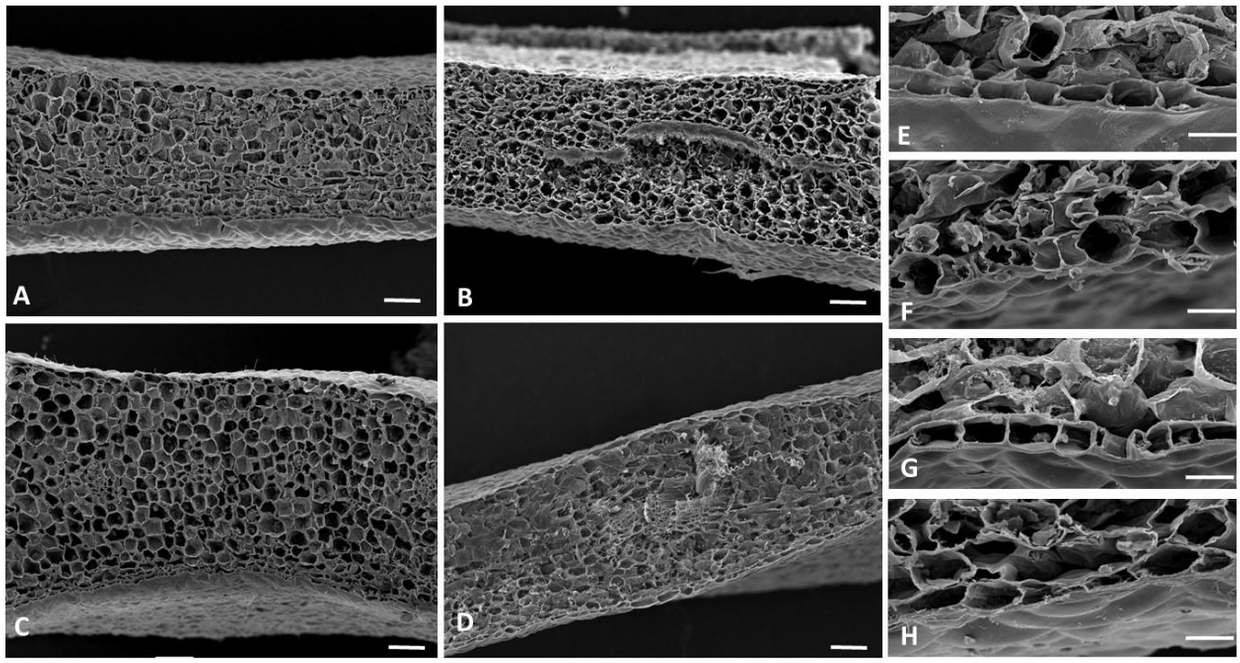

Fig. 8. Scanning electron micrographs of white cabbage leaves: (A–D) cross-sections through the leaf and (E–H) the abaxial epidermis at a higher magnification. The images are from (A and E) control plants, (B and F) plants treated with Mitemine® + Inex-A, (C and G) plants treated with Mitemine® + Inex-A + D-Fense, and (D and H) plants treated with Movento 100 SC. The scale bars represent 100 μm for panels A–D, and 20 μm for panels E–H

erence product Movento 100 SC (containing spirotetramat), which was applied twice during the growing season.

The effect of Mitemine® on insects may be due to the presence of calcium compounds in its formulation and extracts from *A. sativum* (garlic) and *S. indicum* (sesame). The insecticidal and repellent properties of these plant extracts have been demonstrated in several insect species. The insecticidal effect of sesame leaf extract has been reported against *Callosobruchus maculatus* (Fab.) [Negbenebor et al. 2022] and *Clavigralla tomentosicollis* Stål [Negbenebor et al. 2020], whereas garlic essential oil is effective against the adult cereal moth *Sitotroga cerealella* (Olivier) [Yang et al. 2012]. In turn, an ethyl acetate extract from garlic repels the beetles *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* (Motsch.) [Ho and Ma 1995]. These bioactive extracts may act synergistically with calcium compounds to enhance the overall efficacy of Mitemine®, thus providing a comprehensive approach for pest control. The role of calcium as a secondary messenger in multiple signalling pathways to promote both innate immunity and adaptive stress responses in plants has been demonstrated by many researchers [Thor 2019, Parmagnani and Maffei 2022]. Because the phloem is essential for transporting nutrients throughout the plant, it is protected from pests in various ways. Plants defend their phloem primarily through physical barriers and chemical signals. Physical defences prevent insects from reaching nutrients. According to Seki [2016], the resistance of *Dianthus caryophyllus* (L.) to *Tetranychus urticae* (Koch) is related to the thickness of the palisade tissue. Similarly, a tight cell arrangement of mesophyll tissue in savoy cabbage and kale cultivars has been linked to low infestation by *A. proletella* [Marasek-Ciolakowska et al. 2021]. As a result of damage to plant tissues by phloem-feeding insects, there is an increase in calcium ion (Ca^{2+}) levels in plant cells. This leads to the formation of callose, which blocks the phloem tubes and stops the flow of nutrients [Fu et al. 2022]. Moreover, during stress, the Ca^{2+} concentration in plant cells increases, activating

multiple signalling pathways, including those related to jasmonic acid (JA), as well as the activation of genes responsible for lignin synthesis, thereby strengthening cell walls [Denness et al. 2011, Vélez-Bermúdez et al. 2015]. The increased thickness of plant structures we observed in the present study, including the increase in the epidermal and cuticle thickness of vascular veins, appears to be consistent with these mechanisms.

In contrast, insects feeding on phloem sap try to break this line of defence by releasing enzymes and proteins through their saliva. These proteins either degrade defence proteins or bind to Ca^{2+} to inhibit callose synthesis [Will et al. 2013]. Consequently, this can lead to calcium deficiencies in insect foraging areas. It is possible that the use of calcium compounds in our experiment effectively increased the available calcium levels, thereby facilitating the induction of plant resistance to the tested pests.

Modification of the leaf structure, characterised by a decrease in the density and elongation of the stomata occurred after the Mitemine® treatment, may have created an additional barrier to the insects. We have previously confirmed the important role of leaf morphological and anatomical features in the resistance of savoy cabbage (*B. oleracea* var. *sabauda*) and kale (*B. oleracea* var. *sabellica*). Specifically, we found that the cuticle structure, folding of epidermal cells, and stomatal size and density are significant determinants of susceptibility to cabbage whiteflies [Marasek-Ciolakowska et al. 2021].

We also investigated whether adding D-Fense could enhance the effectiveness of Mitemine® in reducing the population of the tested pests. D-Fense contains phosphorus, potassium, an organic *S. alba* extract, and phenolic acids. Phosphorus and potassium, which are essential macronutrients, play a significant role in plant growth. Phosphorus is crucial for energy metabolism, nucleic acid production, cell signalling, and strengthening the cell wall. Potassium regulates water balance by controlling the movement of stomata, stabilising enzymes, and alleviating the effects of stress [Amtmann et al. 2008, Marschner 2012, Sardans and Peñuelas 2021]. Other components of D-Fense are organic white willow extract and phenolic acids, which are thought to have significant biological potential [Deniau et al. 2019]. *Salix alba* extract contains salicin, a precursor of salicylic acid, which plays a significant role in inducing systemic acquired resistance in plants against pathogens. Feng et al. [2021] showed that when applied exogenously, salicylic acid can enhance plant defences against pests with a piercing-sucking mouth apparatus. Under controlled conditions, Ramniwas et al. [2024] showed that an *S. alba* extract reduces feeding and egg laying by oriental fruit fly *Bactrocera dorsalis (Hendel)*, inhibits larval development, and increases the mortality of this insect. The conversion of salicin to salicylic acid may be the key mechanism that triggers resistance in both pathogens and pests. This dual mode of action makes *S. alba* extract a promising alternative to chemical pesticides, especially in the context of sustainable agricultural systems.

Despite the potentially beneficial properties of D-Fens, we found that adding D-Fense to a mixture of Mitemine® and Inex-A did not significantly enhance the effectiveness of pest reduction (Fig. 4A–E). Based on these results, we conclude that the key factor in increasing the resistance of cabbage plants to pests is the combination of Mitemine® and Inex-A.

We found that foliar application of Movento 100 SC, a preparation containing the active substance spirotetramat, had a significant effect on the histological structure of white cabbage leaves, including thickening of the cuticle and epidermis, as well as a reduction in the stomatal density, and an increase in the stomatal length. Spirotetramat belongs to Insecticide Resistance Action Committee (IRAC) group 23. It is distributed systemically in plants and disrupts metabolic processes related to lipid synthesis, which affects the development and reproduction of insects [Brück et al. 2009]. The effect of spirotetramat on the structural characteristics of leaves is surprising, but some studies have indicated that this compound leads to notable physiological and biochemical changes in plants. For example, in cucumbers, spirotetramat increases the activity of antioxidant enzymes, including superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione reductase, and phenylalanine ammonia-lyase [Homayoonzadeh et al. 2022]. Additional biochemical analyses revealed an increase in the content of some amino acids, sucrose, glucose, and fructose. The concentrations of salicylic acid and minerals, such as calcium, manganese, copper, zinc, iron, nitrogen, and magnesium, are elevated in spirotetramat-treated plants. It is worth noting that

salicylic acid can affect the structure of plant tissues, including the structure of the epidermis, the thickness of the cuticle, and the development of the vascular system, because it acts as a phytohormone involved in defence reactions and regulation of plant growth. In tomatoes, Mandal et al. [2009] analysed the effect of exogenous salicylic acid on resistance to pathogens, and found that it strengthens the plant cell wall by increasing lignification. However, the stomatal density may be affected by the nutrient status. Researchers have noted a reduction in the stomatal frequency in lemons [Eichert and Fernández 2023], and a decrease in the stomatal pore sizes and apertures in peaches and pears, as a consequence of iron deficiency [Fernández et al. 2008]. It is possible that the reduction in the stomatal density and the increase in the stomatal length, we observed after applying spirotetramat to the white cabbage plants, may result from the complex physiological and biochemical mechanisms induced by this compound.

Although spirotetramat was the most effective against *B. brassicae*, *A. proletella* larvae, and *P. xylostella* caterpillars, its use is limited to conventional cabbage crops only. Thus, there is a need to search for new products to reduce the occurrence of those pests in both conventional and organic crops. Undoubtedly, the present research using the fertiliser Mitemine® to reduce pests should be classified as an appropriate step in this direction. Regardless of the mechanisms behind the pest population reduction, the inclusion of fertiliser-based products as an alternative to spirotetramat represents a significant step towards sustainable crop protection by reducing environmental impacts and promoting long-term soil and ecosystem health. Although fertilisers can improve plant health and resilience, unlike spirotetramat, they do not provide immediate pest control. Moreover, their use requires more applications, which results in increased direct costs and environmental burdens. Despite these challenges, the use of foliar fertilisers for plant protection against pests remains a valuable approach worth incorporating into modern conservation practices.

CONCLUSIONS

Our 2-years of research using a multi-pest approach has provided robust evidence for the potential of the tested treatments in integrated pest management strategies for cabbage cultivation. Mitemine®, when applied six times with the addition of an adjuvant (Inex-A), reduced the infestation of cabbage plants by *B. brassicae*, *A. proletella*, and *P. xylostella* with effectiveness that was comparable to that of the Movento 100 SC (containing the insecticide spirotetramat), which was applied twice. The addition of D-Fense did not significantly alter the effectiveness of Mitemine®. The results indicate that one of the mechanisms responsible for reducing the pest population density after applying Mitemine® may be thickening of the cuticle and epidermis on the underside of the leaves, including the vascular bundles, which is likely an effect of the calcium present in Mitemine®. We also found that spirotetramat may induce changes in leaf structure similar to those observed after the application of Mitemine®. The observed anatomical modifications offer intriguing insights into the possible mechanisms of induced pest resistance, opening up new avenues for research in plant–pest interactions, and the development of innovative crop-protection strategies. As agricultural systems face increasing challenges from pest pressure and climate change, such multifaceted approaches to crop protection that enhance plant resilience through structural and physiological modifications may play a crucial role in ensuring food security and sustainable agricultural practices.

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CONFLICTS OF INTEREST

The funders (Osadkowski Company) had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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SUSTAINABLE PEST CONTROL: THE ROLE OF CORIANDER AS A REPELLENT PLANT AGAINST *Leptinotarsa decemlineata* SAY IN EGGPLANT CULTIVATION

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ABSTRACT

The study focuses on the potential repellent properties of coriander (*Coriandrum sativum* L.) against the Colorado potato beetle (*Leptinotarsa decemlineata* Say, CPB) when co-cultivated with eggplant (*Solanum melongena* L.). The growth stages of both plants and their relationship to beetle populations were examined. Stage I (Flowering period) and Stage II (Fruit development period) of coriander were shown to have a significant positive influence on reducing the beetle population. Furthermore, a positive correlation was observed between the adult CPB population and Stages I and II of eggplant growth, which are characterised by an increase in flower and fruit numbers when co-planted with coriander. Conversely, a negative correlation was observed between the beetle population and Stages II and III (Fruit maturation period) of eggplant growth in control plots (eggplant-only cultivation). Findings from 2018 and 2021 reinforced the repellent efficacy of coriander, showing a reduction in beetle populations during the coriander flowering, green fruit, and mature fruit stages. These findings indicate that the flowering stage of coriander coincides with the lowest adult beetle densities, suggesting that this phenological phase represents the most effective period for repellent cropping. Future studies should explore the potential role of coriander essential oils in enhancing this effect. This study contributes to the growing body of knowledge on natural pest management strategies.

Keywords: *Coriandrum sativum* L., companion plant, sustainable management, *Solanum melongena* L.

INTRODUCTION

In organic agriculture, which prohibits synthetic fertilizers and pesticides, sustainable pest management practices are essential for maintaining ecological balance and crop productivity [Gamage 2023]. One such practice is companion planting, a system that involves the strategic cultivation of mutually beneficial plant species in proximity. This method not only enhances biodiversity and soil health but also contributes to natural pest suppression by altering pest behaviour, masking host plant volatiles, and attracting natural enemies [Ratnadass et al. 2012].

Among companion plants, aromatic herbs have drawn considerable attention for their bioactive properties. Species such as garlic, rosemary, marigold, and coriander release volatile organic compounds (VOCs) that can repel pests and disrupt their host-finding behaviour. Recent studies have shown that intercropping aromatic plants with main crops, such as tomato, pep-

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per, or cabbage, can reduce pest densities, increase the abundance of beneficial insects, and enhance yields in both rural and urban agricultural systems [Tringovska et al. 2015, Saldanha et al. 2019, Park et al. 2022].

Coriandrum sativum L. (coriander), a member of the Apiaceae family, is increasingly recognised for its pest-repellent activity due to VOCs such as linalool, α-pinene, and camphor. These compounds not only act as olfactory repellents but also exhibit fumigant and contact toxicity against several insect pests [Abid et al. 2019, Elhalawany et al. 2019]. Coriander has also been reported to support populations of natural enemies, further strengthening its role within integrated pest management (IPM) strategies [El-Mogy et al. 2019]. However, field evidence specifically quantifying coriander's effects on the Colorado potato beetle (CPB) under field conditions remains limited [Lenardis et al. 2017, Lee et al. 2018].

A significant threat to solanaceous crops such as eggplant (*Solanum melongena* L.) is the Colorado potato beetle (*Leptinotarsa decemlineata* Say), a highly adaptable pest that causes extensive defoliation and yield loss. The species is notorious for its rapid development of resistance to chemical insecticides, posing challenges to conventional pest control [Wang et al. 2020, Junge et al. 2022]. In this context, there is an urgent need for environmentally sound alternatives that minimise chemical inputs while maintaining pest control efficacy.

Therefore, the present study aims to investigate the potential of *C. sativum* as a natural repellent against the Colorado potato beetle in eggplant cultivation. By assessing the effectiveness of coriander in reducing pest infestation and enhancing crop performance, this research seeks to contribute to sustainable pest management approaches in organic and low-input farming systems. The findings are expected to provide valuable insights for the development of alternative strategies that align with ecological principles while supporting the economic sustainability of vegetable production.

MATERIALS AND METHODS

Plant materials and experimental field

In this study, eggplant variety Pala-49 was used as the main plant, and a small-grained coriander (*Coriandrium sativum* L. microcarpum DC) population of Erzurum/Türkiye origin was used as the repellent plant. Certified seeds of eggplant were obtained from the Atatürk Central Horticulture Research Institute. Coriander seeds were provided by Isparta University of Applied Science, Faculty of Agriculture, Department of Field Crops, Herbs Garden. The fruit diameter of the coriander seeds used in the experiment was 3 mm, and the weight of 1000 grains was 7 g. The experiment was conducted in an organic plot in Ürünlü village, Nilüfer, Bursa, which holds an organic certification and is situated at an approximate elevation of 451 meters above sea level.

Experimental design and cultivation methods

The experiment was established with three replications. The total experimental area was 60 m², consisting of three plots, each measuring 20 m² in size. Prior to plot establishment, approximately 50 kg of well-decomposed farmyard manure was applied to each 20 m² plot, totalling 150 kg for the entire area. Following the application of manure, the plots were tilled using a rotary hoe, and surface levelling was performed to prepare the soil for sowing. The preceding crop grown in the experimental field was faba bean (*Vicia faba* L.), which is known to contribute to soil nitrogen content due to its ability to fix nitrogen symbiotically. The plants used in the experiment were obtained through different methods: coriander and eggplant seeds were propagated separately. While coriander seeds were directly sown by hand into the field plots, eggplant seeds were germinated in seedling trays under greenhouse conditions and grown into transplants.

Eggplant seeds were sown on March 10 under controlled greenhouse conditions for later transplanting into the field. The seeds were sown into 48-cell plastic trays filled with a well-drained, chemical-free, and organically certified peat-based growing medium. The trays were kept in a polyethene-covered greenhouse, where the daytime temperature was maintained at 25 ±2 °C and the nighttime temperature at 18 ±2 °C, with a relative humidity range of 65% to 75%. A photoperiod of approximately 14–16 hours was maintained using natural daylight, supplemented with artificial lighting as needed. No chemical fertilizers or pesticides were used during the seedling stage. Instead, seedlings were fed twice at 10-day intervals using a liquid manure tea applied via root irrigation.

The manure tea was prepared by diluting well-decomposed farmyard manure at a ratio of 1 : 6 with water that had been left to stand for two days, allowing chlorine to evaporate and sediments to settle. When the seedlings were approximately 30–35 days old, they were transplanted into the field.

Coriander seeds were manually sown at a rate of 15 kg per hectare on May 10, 2018, for the first year. However, the experiment was paused from 2019 to 2020 due to the COVID-19 pandemic. The trials resumed in 2021, with coriander sown again on May 17. Irrigation of coriander plants was conducted at regular intervals, depending on the growth stage and environmental conditions. Surface irrigation was applied using a hose to ensure uniform water distribution across the plots. Irrigation frequency was determined based on soil moisture levels, and water stress was minimised.

When the coriander plants and eggplant seedlings reached similar growth stages, the eggplant seedlings were transplanted into the centre of plots surrounded by coriander plants, as well as into control plots without coriander. Coriander plants were densely sown along the borders of the plots, forming a hedge-like barrier. Eggplants were planted with a spacing of 80 × 100 cm between and within rows. Each replication included four rows with 16 eggplant plants. A total of 96 eggplant plants were used in the experiment, 48 in the coriander treatment and 48 in the control plots. The spacing between the eggplants and their distance from the surrounding coriander plants were kept uniform, approximately 100 cm.

Pest monitoring and phenological observations

Regular cultural practices were carried out for eggplant and coriander in the companion planting, while regular observations were made. While conducting adult counts, phenological observations of eggplant and coriander were also performed and recorded as follows: the period when flowering (F) begins (Stage I); the period when green fruit (GF) were observed (Stage II); the period when mature fruit (MF) were seen (Stage III) [Martínez-Ispizua et al. 2021]. The percentage of plants in the individual development stages presented in Table 1–2 was calculated as the ratio of the number of plants exhibiting the mentioned phenology stage to the total number of plants. During the study, potato beetle eggs and larvae were encountered; however, this research focused on the adults that had emerged after wintering and were heading towards the eggplants, as well as the newly developing offspring. Sampling of CPB adults took place when the eggplants reached stages I–III, with the sampling occurring close to the same time – between 9:00 and 11:00 am, when insect activity is high and the heat of the day has not yet built up. A sample of 4 plants per row was used. Insect

Table 1. CPB count dates and corresponding eggplant and coriander phenology in 2018

Weeks	Count dates	Eggplant phenology	Stage	Coriander phenology	Stage
1W	July 6	10% F	I	50% F	I
2W	July 13	25% F	I	85% F, 5% GF	I–II
3W	July 20	40% F, 10% GF	I–II	50% F, 30% GF	I–II
4W	July 27	60% F, 20% GF, 20% MF	I–II–III	25% F, 70% GF, 5% MF	I–II–III
5W	August 3	50% F, 30% GF, 20% MF	I–II–III	5% F, 80% GF, 15% MF	I–II–III
6W	August 10	50% F, 30% GF, 20% MF	I–II–III	55% GF, 35% MF	II–III
7W	August 17	30% F, 20% GF, 50% MF	I–II–III	45% GF, 55% MF	II–III
8W	August 24	20% F, 10% GF, 70% MF	I–II–III	25% GF, 75% MF	II–III
9W	August 31	5% F, 10% GF, 85% MF	I–II–III	100% MF	III
10W	September 7	100% MF	III	100% MF	III

F – flowering, GF – green fruit, MF – mature fruit

Table 2. CPB count dates and corresponding eggplant and coriander phenology in 2021

Weeks	Count dates	Eggplant phenology	Stage	Coriander phenology	Stage
1W	July 16	35% F	I	65% F, 15% GF	I–II
2W	July 23	40% F, 10% GF	I–II	70% F, 30% GF	I–II
3W	July 30	50% F, 20% GF,10% MF	I–II–III	50% F, 30% GF, 20% MF	I–II–III
4W	August 6	40% F, 30% GF, 30% MF	I–II–III	20% F, 50% GF, 30% MF	I–II–III
5W	August 13	20% F, 30% GF, 50% MF	I–II–III	5% F, 40% GF, 55% MF	I–II–III
6W	August 20	20% F, 30% GF, 50% MF	I–II–III	25% GF, 75% MF	II–III
7W	August 27	5% F, 15% GF, 80% MF	I–II–III	100% MF	III
8W	September 3	15% GF, 85% MF	II–III	100% MF	III
9W	September 10	100% MF	III	100% MF	III
10W	September 17	100% MF	III	100% MF	III

F – flowering, GF – green fruit, MF – mature fruit, stage I – flowering period, stage II – fruit development period, stage III – fruit maturation period

counts were performed by counting specimens directly on the plants. The same methodology was used on the control plots. Data was collected over 10 weeks. Counts of the CPB adults were conducted weekly and recorded. No essential oils were extracted or applied; our field trial exclusively evaluated coriander–eggplant intercropping.

Data analysis

In this research, multiple linear regression analysis was utilised to examine the relationships between the adult population of *L. decemlineata* and the phenological stages of eggplant and coriander plants. The analysis of the data collected from 2018 and 2021 involved fitting a linear equation, with the adult population as the dependent variable and plant stages as independent variables. P-values less than 0.05 indicated statistically significant associations. An influence plot was generated to identify influential points and potential outliers, ensuring the robustness of the analysis. All statistical analyses were conducted using JMP software (version 7.0; SAS Institute Inc., Cary, NC, USA).

RESULTS

In 2018 and 2021, the adult population of *L. decemlineata* was monitored and documented weekly for both years, in relation to the phenological development of eggplant and coriander plants (Tab. 3).

In 2018, in plots where coriander and eggplant were planted together, the CPB adult population and plant-related ‘Stage I’, ‘Stage II’, and ‘Stage III’ data are shown in Figure 1. In plots where only eggplant was planted as a control plot, the CPB adult population and plant-related ‘Stage I’, ‘Stage II’, and ‘Stage III’ data are shown in Figure 2. The multiple linear regression analysis revealed a statistically significant positive relationship between the CPB adult population and the ‘Stage I’ and ‘Stage II’ data of the eggplant, planted together with coriander, with a p-value less than 0.05. The p-values for ‘Stage I’ and ‘Stage II’ data were 0.0118 and 0.0127, respectively. As the number of flowers and fruits increases, the CPB adult population tends to increase as well (Fig. 3A). The multiple linear regression analysis revealed a statistically significant negative relationship between the CPB adult population and the ‘Stage II’ and ‘Stage III’ data of the eggplant that was planted as a control, with a p-value less than 0.05. The p-values for ‘Stage II’ and ‘Stage III’ data were 0.0008 and 0.0390, respectively. Eggplants co-planted with coriander exhibited an increase in CPB adult number corresponding with ‘Stage I’ and ‘Stage II’ growth (Fig. 3A), whereas on the control eggplants, a decline in CPB adult number during ‘Stage II’ and ‘Stage III’ has been noted (Fig. 3B).

During the initial four-week period of 2018, observations indicate a reduced population density of adult CPB in plots incorporating both coriander and

Table 3. Phenology of eggplant and coriander, and *Leptinotarsa decemlineata* adult population in 2018 and 2021

Plant	Week	2018				2021			
		Stage I (%)	Stage II (%)	Stage III (%)	CPB (average adult number per)	Stage I (%)	Stage II (%)	Stage III (%)	CPB (average adult number per)
Coriander	1W	50	–		–	66	17	–	–
	2W	87	7	–	–	71	31	–	–
	3W	51	31	–	–	52	31	21	–
	4W	26	71	6	–	21	52	32	–
	5W	8	82	17	–	7	41	56	–
	6W	–	57	37	–	–	25	76	–
	7W	–	46	57	–	–	–	98	–
	8W	–	27	76	–	–	–	99	–
	9W	–	–	99	–	–	–	99	–
	10W	–	–	99	–	–	–	100	–
Eggplant co-planted with coriander	1W	11	–	–	*0.03*	35	–	–	*1.38*
	2W	27	–	–	*0.72*	42	11	–	*3.34*
	3W	42	8	–	*0.76*	52	21	12	*0.07*
	4W	62	22	21	*0.03*	41	32	31	*0.69*
	5W	52	31	22	*5.38*	23	32	52	*0.34*
	6W	51	31	22	*1.38*	22	31	52	*1.34*
	7W	31	21	51	*0.03*	8	16	82	*0.07*
	8W	20	11	72	*9.39*	–	17	86	*0.00*
	9W	6	10	88	*0.03*	–	–	99	*0.03*
	10W	–	–	99	*0.69*	–	–	100	*0.07*
Eggplant as a control	1W	12	–	–	*1.03*	37	–	–	*4.00*
	2W	27	–	–	*3.34*	42	10	–	*2.39*
	3W	42	11	–	*8.72*	52	21	12	*0.00*
	4W	62	32	22	*7.03*	42	31	31	*1.07*
	5W	62	31	23	*4.39*	22	33	52	*6.71*
	6W	51	31	21	*2.32*	22	32	51	*7.00*
	7W	32	21	52	*2.03*	6	16	81	*0.07*
	8W	22	11	70	*0.34*	–	17	81	*0.07*
	9W	6	12	86	*0.38*	–	–	99	*0.00*
	10W	–	–	99	*0.03*	–	–	100	*0.07*

Stage I – flowering period, stage II – fruit development period, stage III – fruit maturation period

eggplant (Fig. 1). In contrast, control plots featuring solely eggplant (Fig. 2) exhibited a notable peak in adult population during the third and fourth weeks, averaging approximately 7 to 8.7 individuals per week. The suppressed population levels in the co-cultivation plots of coriander and eggplant could potentially be attributed to the potent repellent properties exhibited by coriander during its flowering and green fruit developmental stages against adult CPB. By the eighth week, the control plot suffered significant leaf damage instigated by the beetles, leading to their subsequent departure and a reduction in the overall adult beetle population. Interestingly, a surge in the adult beetle population was observed during the eight weeks in the plots co-cultivated with coriander and eggplant. This observation could potentially suggest a diminished repellent efficacy of coriander coinciding with an escalation in the quantity of mature fruits present on the plant.

In 2021, in plots where coriander and eggplant were planted together, the adult population and plant-related data for 'Stage I', 'Stage II', and 'Stage III' are shown in Figure 4. In plots where only eggplant was planted, the CPB adult population and plant-related 'Stage I', 'Stage II' and 'Stage III' data are shown in Figure 5. The multiple linear regression analysis revealed a statistically significant negative relationship between the CPB adult population and the 'Stage III' data of the eggplant, with a p-value less than 0.05 (P = 0.04619). An increase in 'Stage III' data tends to decrease the CPB adult population (Fig. 6A). The multiple linear regression analysis revealed a statistically significant positive relationship between the CPB adult population and the number of fruits in the eggplant, with a p-value less than 0.05 (P = 0.0254) – as in Figure 6B. In 2021, the control plots consisting only of eggplants (Fig. 5) recorded a peak in CPB adult population during the fifth and sixth weeks, with an approximate weekly average of 7 individuals. Interestingly, the adult population in the co-cultivation plots of coriander and eggplant (Fig. 4), where 70% of flowering occurred, was notably lower (0.5 –1 adult) compared to the control plots. Specifically, during the fifth week,

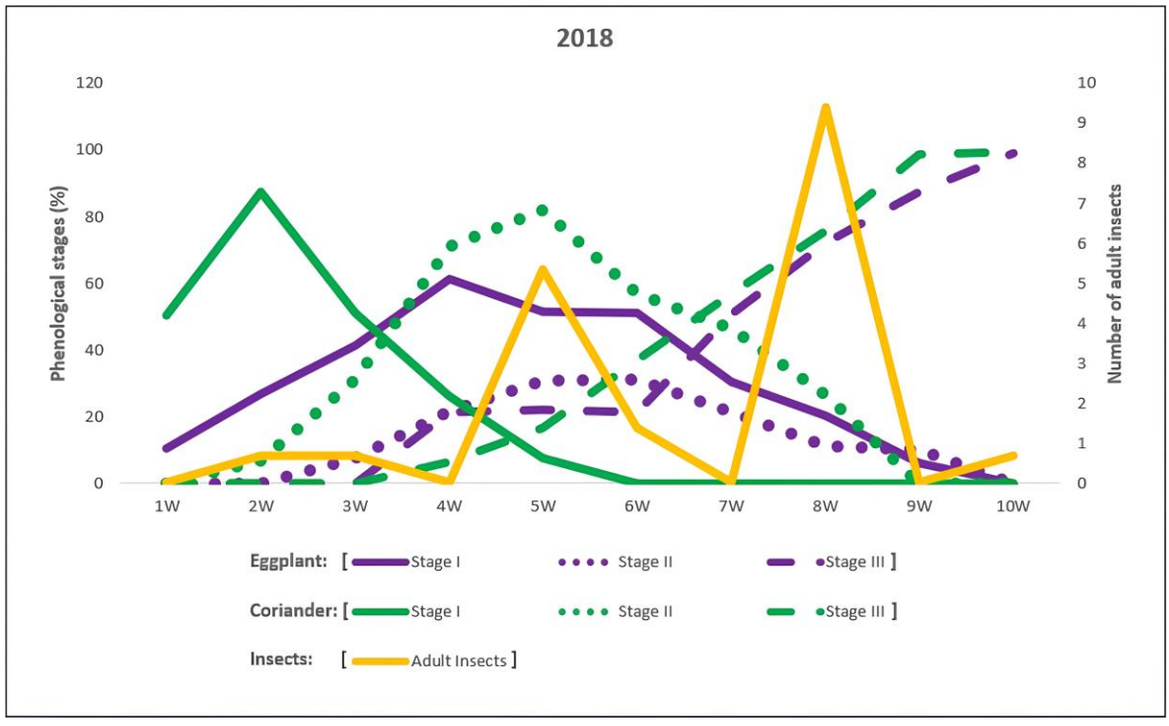

Fig. 1. *Leptinotarsa decemlineata* adult population and plant parameters comparison in coriander and eggplant co-cultivation in 2018. Stage I – flowering period. Stage II – fruit development period. Stage III – fruit maturation period

when 7% flowering, 41% green fruit, and 56% mature fruit stages were observed in the co-cultivation plots, the adult beetle population was significantly reduced (approximately weekly average of 0.3 individuals), as opposed to the relatively high population in the control plots. By the sixth week, despite the presence of 25% flowering and 76% mature fruit stages in the co-cultivation plots, the adult beetle population remained low, whereas it experienced a peak in the control plots (approximately a weekly average of 7 individuals).

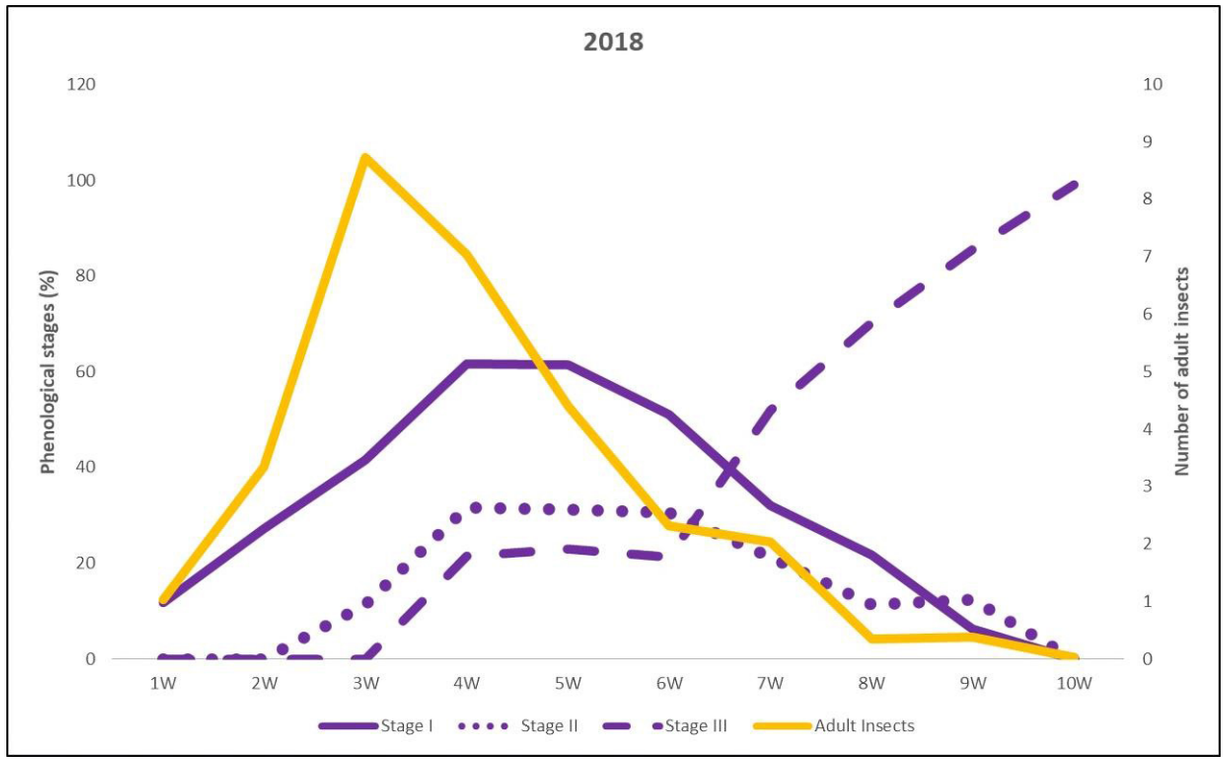

Fig. 2. *Leptinotarsa decemlineata* adult population and plant parameters comparison in eggplant-only control plots in 2018. Stage I – flowering period. Stage II – fruit development period. Stage III – fruit maturation period

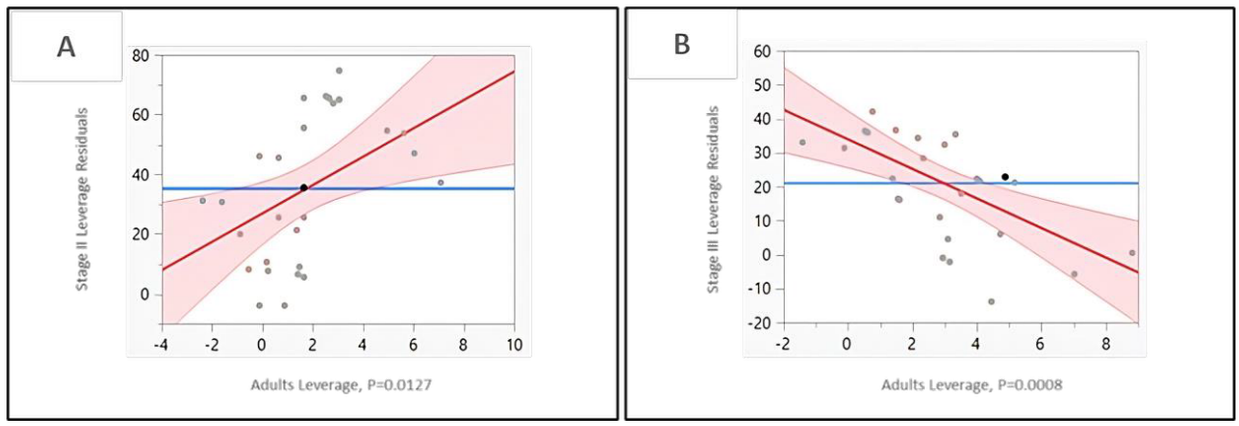

Fig. 3. Relationship between the Colorado potato beetle adult population and host plant developmental stage in 2018. (A) Coriander and eggplant co-cultivation, (B) Eggplant-only control plots. In both cases, an influence plot based on leverage and residual values is presented to identify influential observations and potential outliers

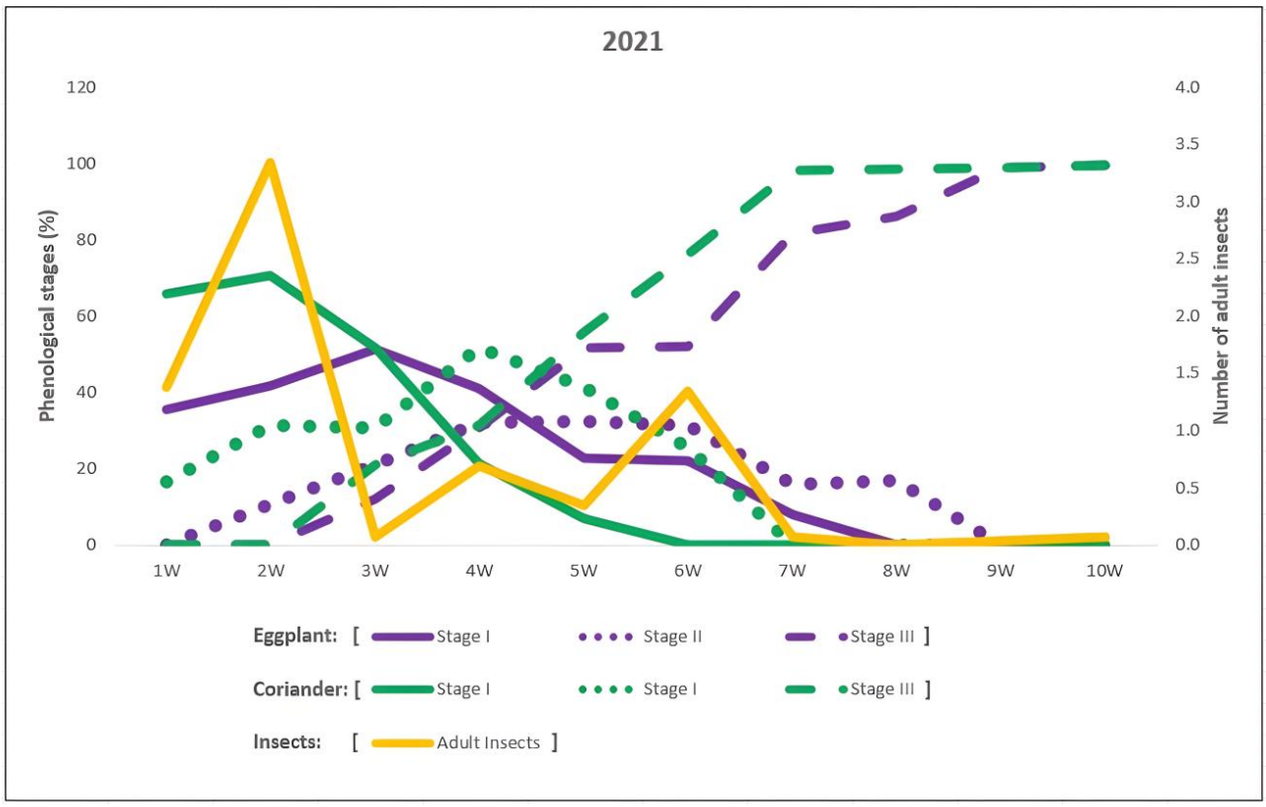

Fig. 4. *Leptinotarsa decemlineata* adult population and plant parameters comparison in coriander and eggplant co-cultivation in 2021. Stage I – flowering period. Stage II – fruit development period. Stage III – fruit maturation period

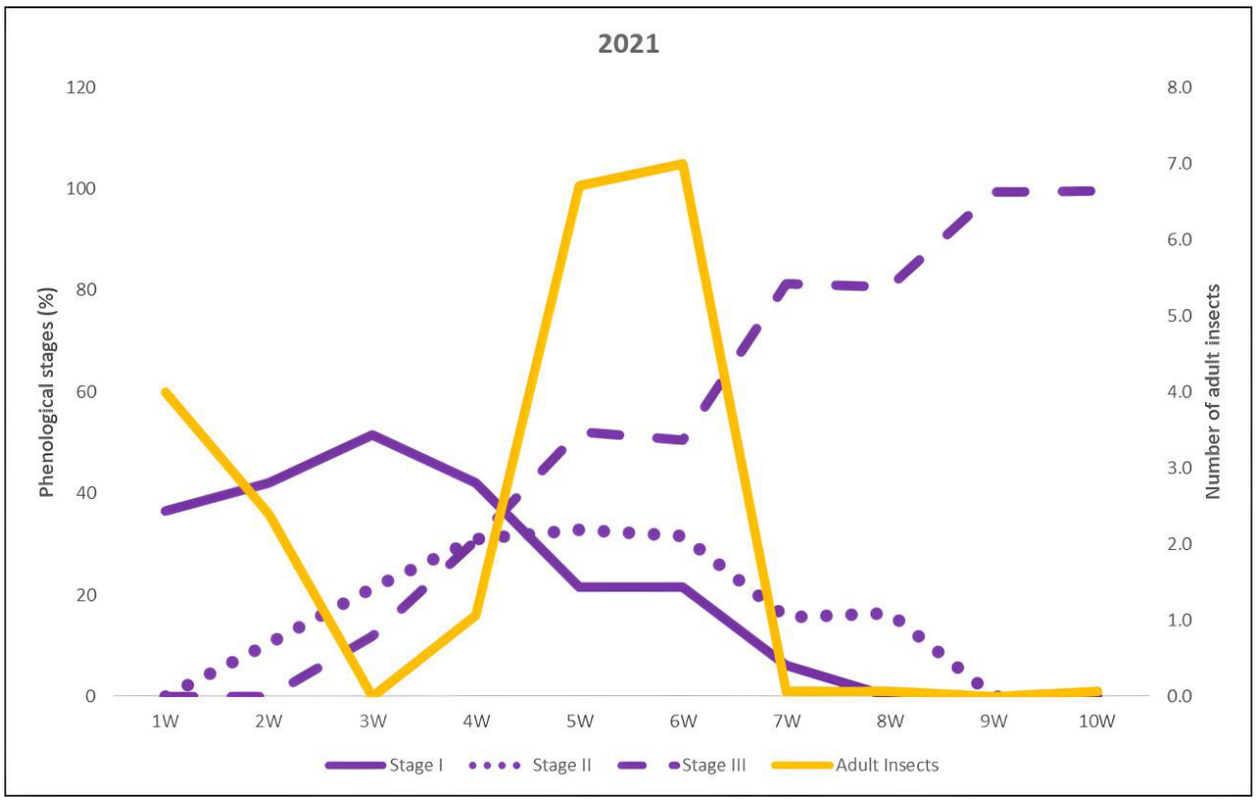

Fig. 5. *Leptinotarsa decemlineata* adult population and plant parameters comparison in eggplant-only control plots in 2021. Stage I – flowering period. Stage II – fruit development period. Stage III – fruit maturation period

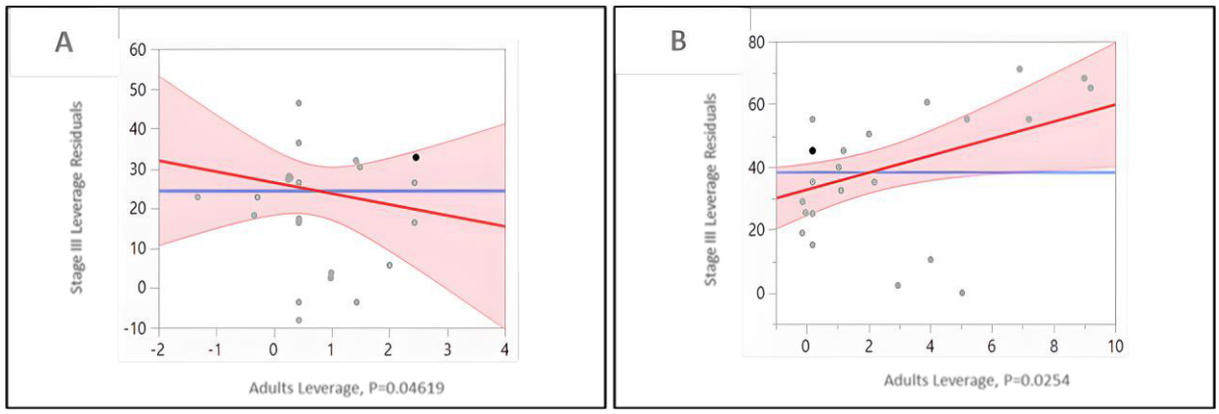

Fig. 6. Relationship between the Colorado potato beetle adult population and host plant developmental stage in 2021. (A) Coriander and eggplant co-cultivation, (B) Eggplant-only control plots. In both cases, an influence plot is presented to show influential observations and potential outliers

Considering this set of data, it can be inferred that the flowering, green fruit, and mature fruit stages of coriander manifest notable repellent properties.

DISCUSSION

Based on reports that aromatic companion plants (including coriander) can repel herbivores, we evaluated a coriander–eggplant intercrop as a VOC-mediated tactic against *L. decemlineata* under field conditions [Asare-Bediako et al. 2010, Khafagy et al. 2020].

Several scholarly studies have explored the potential of diverse plant species, including those characterised by aromatic properties, for pest management. Yousef et al. [2024] undertook a comparative meta-analysis focusing on three essential crops (wheat, maise, and soybean) to investigate how diversification schemes infield (intercropping) and agri-environmental schemes (flower strips, hedgerows and field margins) affect arthropod abundance. In contrast, monoculture systems, which rely on a single crop, negatively impact natural enemies and increase the populations of pests. These findings suggest that diversified farming practices can be an effective strategy to promote a healthy and sustainable insect community in the analysed cropping systems. Although the study did not focus on coriander, it offers important insights into the potential implications of diversified farming practices for insect communities in this crop group. Consistent with this, we observed lower adult CPB at key stages in coriander–eggplant plots versus eggplant-only controls, whereas in 2021, adult counts in controls increased with fruit number ($p \leq 0.05$), and the fruit–beetle association is interpreted as phenological co-variation.

This pattern is plausibly consistent with stage-dependent variation in coriander essential oil content. Previous studies indicate that essential oil concentration increases from approximately 0.23% at full flowering to 0.37% during the green fruit stage, and peaks at up to 2.5% in mature fruit stages [Telci et al. 2006, Ramezani et al. 2009]. These periods coincided with the weeks in which CPB adults were most reduced in our mixed plots. Although coriander essential oil and its constituents exhibit bioactivity in laboratory assays, their composition and efficacy vary with the plant organ and phenological stage, as well as the extraction protocol. These sources of variability limit standardisation and complicate on-farm "homemade" preparations. Our study, therefore, focused on living-plant intercropping rather than oil applications; future work should benchmark field-rate EO sprays under standardised extraction/formulation before agronomic recommendations are made.

The present study's findings on the impact of intercropping coriander and eggplant on the CPB adult population align with previous research that demonstrates the efficacy of intercropping and companion planting in pest management. Kheam et al. [2024] found that volatile organic compounds emitted by specific plant species can alter insect behaviour and reduce coloni-

sation of primary host plants, which may contribute to the observed decrease in the *L. decemlineata* adult population in the coriander-eggplant intercropping system. Moreover, Afrin et al. [2017] reported that intercropping mustard with coriander significantly reduced pest infestations while preserving honeybee populations, suggesting the potential of coriander as a companion plant for pest management. Breitenmoser et al. [2022] emphasised the importance of choosing suitable intercropping species to manage pest populations and promote biodiversity in winter oilseed rape agroecosystems. Similarly, Tajmiri et al. [2017] found that strip-intercropping potatoes with annual alfalfa successfully managed Colorado potato beetle populations and increased the presence of natural predators. These findings support the notion that intercropping can help control *L. decemlineata* adult populations when suitable companion plants are used.

In support of this, the dominant essential oil compounds identified in coriander, such as linalool (up to 87.5%), α-pinene (up to 10.9%), camphor (up to 5.1%), and γ-terpinene (up to 11.2%) are all known to exert neurotoxic or deterrent effects on insect pests [Pande et al. 2010, Mageed et al. 2012, Freires et al. 2014]. These volatiles likely interfere with pest orientation and host detection, contributing to the reductions observed in CPB populations.

Alioghli et al. [2022] reported that specific potato-safflower intercropping patterns effectively managed Colorado potato beetle populations and improved crop yields. This finding further corroborates the potential of well-designed intercropping systems for pest management, as observed in the present study. Lastly, Ben-Issa et al. [2017] highlighted the importance of selecting appropriate companion plants based on their interactions with the target pest and compatibility with the main crop. The current study demonstrates the potential of coriander as a companion plant for eggplant to mitigate *L. decemlineata* adult populations, contributing to the growing body of evidence supporting the use of companion planting for sustainable pest management in agricultural systems.

CONCLUSIONS

The observed results demonstrate that co-cultivating coriander and eggplant significantly influences the CPB adult population, highlighting the importance of considering these plant species during cultivation. Further investigation into the underlying causes and mechanisms of these relationships is warranted, as this could potentially contribute to the advancement of more efficacious strategies in horticultural practices and integrated pest management. In summary, trials conducted in 2018 and 2021 revealed that the number of adult CPB decreased during both the flowering and fruit development stages of coriander. It could potentially be due to the presence of essential oils in coriander. Further research is necessary to understand better the potential role that these essential oils play in reducing the CPB population. Further studies should also be undertaken to determine the presence and composition of secondary metabolites in companion plants based on their growth stages.

CONTRIBUTIONS

S.B., O.K., N.S.G. reviewed and edited the draft. All authors have read and approved the final version to be published.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

FUNDING

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AVAILABILITY OF DATA AND MATERIAL

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors affirm that they have no recognisable financial interests or personal relationships that could have impacted the research findings presented in this manuscript.

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EFFECTS OF NITROGEN DOSE AND N-NH_4:N_{total} RATIO ON GROWTH, YIELD, AND QUALITY OF GREENHOUSE LETTUCE ACROSS SEASONS

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ABSTRACT

A factorial experiment testing the effects of different nitrogen dose applications (60, 80, and 120 kg ha^{-1}) provided varying ratios of N-NH_4 to the total amount of nitrogen supplied (0.4, 1.0) was conducted in two successive growing seasons (autumn-winter and spring) with the *Lactuca sativa* Lagarde F1. Phosphorus and potassium fertilizers were kept constant and uniform at all experimental plots, respectively 25 (P_2O_5) and 180 (K_2O) kg ha^{-1}. Root traits, growth parameters, yield, nitrogen use efficiency (NUE), and NO_3 concentration in the lettuce leaves were measured and analyzed. N-NO_3 concentration and NUE were the most sensitive traits to N dose applications and N-NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to seasonal variation of environmental factors. A range of 60–80 kg ha^{-1} N was the optimum for greenhouse lettuce fertilization. Further increase of N dose applications did not provide a higher yield, whereas it significantly increased the N-NO_3 concentration in the plant and reduced the N use efficiency. The NO_3 concentration in the lettuce leaves was reduced by increasing the ratio of N-NH_4 to total N applied and extending the period of the latest N application before harvesting.

Keywords: *Lactuca sativa*, root traits, nitrogen use efficiency, NO_3 concentration, fertilization

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops, occupying a total of 1226,370 ha and reaching a worldwide production of 27,660,187 Mt [Martínez-Moreno et al. 2024]. It is an important source of vitamins and minerals and of bioactive compounds such as polyphenols, carotenoids, and chlorophyll, with significant health benefits [Shi et al. 2022, Martínez-Moreno et al. 2024]. Yet, similar to other raw, salad-type crops, lettuce is considered to be one of the main sources of dietary nitrate intake [Blekkenhorst et al. 2017].

Although there are several benefits of nitrate on human health [Du et al. 2007], its potential harmful effects are a common public concern. Nitrates themselves have low toxicity to humans, but nitrates become reduced to nitrites, which react with other compounds as amines or amino acids contained in food, leading to nitrosamine formation [Urlić et al. 2017b, Martínez-Moreno et al. 2024]. These specific compounds might put humans at risk of gastrointestinal cancer and methemoglobinemia [Liu et al. 2016]. The risk is highlighted in EU Regulation 1258/2011, which sets upper limits on nitrate levels in lettuce depending on the growing season: 5000 mg kg^{-1} fresh mass (FM) in winter and 4000 mg kg^{-1} FM in summer for greenhouse lettuce [European Commission 2011]. Hence,

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reducing nitrate content in vegetables through modulating the production environment is a major concern of lettuce producers [Liu et al. 2016].

Nitrate is taken up by roots and stored in root vacuoles or is transported to leaves, where it is reduced to ammonium and incorporated into carbon skeletons, organic molecules that act as acceptors for reduced nitrogen during amino acid biosynthesis, or otherwise stored in the leaves [Castaings et al. 2011]. The absorption, translocation, and assimilation of nitrates in vegetables are tightly regulated by the interaction of internal cues and external environmental factors [Bian et al. 2020]. The amount and form of N-fertilizer, light availability and quality, water availability, air and root zone temperature, and CO_2 concentration [Bian et al. 2020, Martínez-Moreno et al. 2024] affect N uptake and accumulation in plants. Among them, nitrogen fertilization and light intensity are the most important factors.

Nitrate accumulation in leafy vegetables is tightly linked to N fertilization practices. Various studies reported a significant positive correlation between the dose of nitrogen supply and NO3 content in leafy vegetables [Fu et al. 2017, Ortega-Blu et al. 2020]. As a common rule, an increase in N supply is followed by a significant increase in N concentration [De Pinheiro Henriques 2000, Cometti et al. 2011, Fu et al. 2017]. Furthermore, N fertilization and the type of N fertilizers impact the fresh yield and might pose some environmental risks. Over-fertilization limits lettuce productivity because of osmotic stress [Albornoz and Lieth 2015], whereas high-dose N applications are responsible for significant N losses and severe environmental impacts [Balliu et al. 2007a, 2008].

Appropriate NH_4:NO_3 ratios can modulate N accumulation in the plant's leaves [Hachiya and Sakakibara 2017]. An increase in NH_4 supply versus the total amount of N supplied to the plants leads to a reduction in N-NO_3 accumulated in the plants [Santamaria et al. 2001]. Next to it, the NH_4:NO_3 ratio impacts the morphology of the root system. It has been proven that ammonium supply increases lateral root initiation and higher-order lateral root branching, whereas the elongation of lateral roots is stimulated mainly by nitrate [Lima et al. 2010, Hachiya and Sakakibara 2017].

Light intensity modulates uptake and nitrate reduction, and is considered the leading environmental factor controlling nitrate accumulation in leafy vegetables [Albornoz and Lieth 2015]. Plants reduce biomass production [De Pinheiro Henriques 2000], but accumulate more nitrate under low light intensities [Lillo and Appenroth 2001]. Furthermore, significant interactions between N supply and light intensity are often reported in lettuce crops and other leafy vegetables. An increase in N supply, combined with reduced light intensity, is the recipe for having the maximum N-NO_3 concentration in the plant [Cometti et al. 2011, Fu et al. 2017]. Still, besides the intensity, the quality of light plays a significant role in the N accumulation in lettuce plants [Chen et al. 2014, Liu et al. 2016].

Considering that background, the goal of this experiment was to estimate the potential interactions between N dose applications and different NH_4-N:total-N ratios as they were modulated by the growing season, on root morphology traits, N use efficiency, yield, and NO_3 concentration of lettuce plants grown under greenhouse conditions.

MATERIALS AND METHODS

Plant materials and experimental design. The study was conducted in plastic, non-heated greenhouses, Vushtrri municipality (42°53'N, 20°52'E) of the Republic of Kosovo. The area is characterized by a continental climate with temperatures varying from 0.6 to 22.4 °C, whereas the irradiation values range from 42 to 214 kWh. Details of the climatic conditions during both growing conditions are provided in the Table 1. Natural soil classified as clay loam soil was used as growing media. Details about soil physical and chemical characteristics are provided in Table 2. Two sequential planting seasons, autumn (October 2023–February 2024) and spring (February–May 2024), have been experimented with.

The lettuce cultivar Lagarde F1 (BASF) was used as a plant material in both seasons. A split-plot experimental design was established with N doses (respectively 60, 80, and 120 kg ha^{-1}), as the main factor, and N-NH_4 to total nitrogen supplied (N_{total}) ratio (0.4, 1.0) as the secondary factor. Phosphorus and potassium were kept constant and uniform at all experimental plots, respectively 25 (P_2O_5) and 180 (K_2O) kg ha^{-1}. An experimental unit (treatment) was represented by a 2 square meter plot (2 m × 1 m). Thirty-day-old seed-

Table 1. The average monthly temperatures and global horizontal irradiation. Data retrieved from PVGIS. European Union. https://re.jrc.ec.europa.eu/pvg_tools/en/tools.html

Month	Average temperature (°C)	Global horizontal irradiation (kWh)
January	0.6	47.8
February	2.3	65.9
March	5.8	107.5
April	10.8	145.7
May	15.0	179.4
June	19.6	198.1
July	22.2	214.3
August	22.4	193.6
September	17.2	134.5
October	11.6	95.6
November	6.6	57.0
December	2.0	41.6

Table 2. The physical and chemical properties of the soil

The parameters and methods				
Volumetric density (g cm^{-3})	Granulometric analysis (%)			
	Sand (2.0–0.2 mm)	Fine sand (0.2–0.02 mm)	Silt (0.02–0.002 mm)	Clay (<0.002 mm)
2.50	3.0	29.8	25.2	27.6
pH (H_2O)	$CaCO_3$ (%)	organic matter (%)	nitrogen (%)	Ass. P_2O_5 (mg 100g)
7.04	2.78	2.94	0.14	14.7
1:2 method S-2.10	Volumetric method	SPWR 2003 Method S-14.10	Soil Kjeldahl Nitrogen Method S-8.10	Mehlich 3
Exchangeable macro nutrients (mg kg^{-1})				
Ca	Mg	K	Na	S
1743	151	134	12	147
Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3
Micronutrients (mg kg^{-1})				
B	Zn	Fe	Mn	Cu
0.19	2.43	0.57	0.12	0.85
Hot water	Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3

lings were transplanted in the field at a fixed planting density of 24 m^{-2} (0.25 m × 0.25 m). Four randomly distributed replications were applied for each experimental unit, assembling in total a 24-treatment design (3 (N doses) × 2 (NH_4:N_{total} ratios) × 4 (replications)).

Combinations of different fertilizers: Frutta (Adriatica Spa, Strada Dogado, Rovigo, Italia; 8-16-20--13.5-11), K_2SO_4 (0-0-51), NH_4NO_3 (34-0-0), and $CO(NH_2)_2$ (46-0-0) at different amounts were used to compose the specific nutrient formulations for each treatment. Details on the respective fertilizer compositions were provided in Table 3. The whole amount of Frutta (8-16-20-13.5-11) was applied as basic fertilization, providing that the total amount of phosphorus and partial amounts of potassium and nitrogen were applied before transplanting. The rest of the fertilizers were delivered during the vegetation and split into two equal doses for each specific treatment. Common

Table 3. Detailed information on the fertilizer's recipe composition

	A_60-25-180							
	Fertilizer's composition	Amount of each fertilizer used (kg ha^{-1})	N-total	N-NO_3	N-NH_4	P_2O_5	K_2O	NH_4:N_{Total} ratio
A1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	140	47.5	36.4	12.5			
	Total A1		**60**	**36**	**25**	**25**	**180**	**0.4**
A2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	103	47.5		47.5			
	Total A2		**60**	**0**	**60**	**25**	**180**	**1.0**
	B_80-25-180							
B1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	198	67.5	51.7	17.8			
	Total B1		**80**	**52**	**30**	**25**	**180**	**0.4**
B2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	147	67.5		67.5			
	Total B2		**80**	**0**	**80**	**25**	**180**	**1.0**
	C_120-25-180							
C1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	316	107.5	82.3	28.3			
	Total C1		**120**	**82**	**41**	**25**	**180**	**0.4**
C2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	234	107.5		107.5			
	Total C2		**120**	**0**	**120**	**25**	**180**	**1.0**

commercial crop management practices were equally applied to all treatments during the plant life cycle. Harvesting was conducted when the plant's rosette reached marketable size, respectively 107 days after transplanting (DAT) in the autumn season, and 68 DAT in the spring season. The number of marketable heads was counted for each treatment and each replication, and the average head weight was calculated as the ratio of total weight to the number of respective heads for each replication.

Nitrate/nitrite measurements. Mature leaves of ten randomly selected plants in each experimental plot were collected to analyze the nitrate (NO_3) and nitrite (NO_2) concentrations. The leaves were washed out carefully, dried, and analyzed by the UV-VIS spectroscopy method [Cataldo et al. 1975, Zhao and Wang 2017] using a Spectrophotometer (NANOCOLOR VIS II). The analyses were performed only at the harvesting time, in the autumn season, whereas several successive measurements (5, 12, and 34 days after the latest fertilizer application) were performed during the spring season.

Biomass assessment and root analyses. On the harvesting day, six plants were randomly selected and used for biomass assessment and root morphology analyses. For that purpose, the plants were carefully removed from the soil with their root system intact. The roots were dissected from the aboveground organs, washed free of adhering soil particles using a soft water jet, and scanned with an Epson Expression/STD 4800 Scanner. The acquired root images were analyzed with WinRHIZO Arabidopsis 2013 software (Regent Instruments Inc., Quebec, Canada). Root length (RL), root surface area (RSA), average root diameter (AvgD), and root volume (RV) were individually measured and recorded.

The leaves were carefully removed from the rosettes and individually counted for each plant. Ten cm^2 discs were cut from 10 randomly selected leaves of each treatment. They were dried out for 72 h at 65 °C and weighed with an accuracy of ±1 mg. The coefficient of leaf area per unit of dry weight (cm^2 mg^{-1}) was calculated for each treatment, which was then used to calculate the entire leaf area for each treatment.

Following root morphology analyses, the roots and leaves of each plant were dried (65 °C, 72 h), and the dry matter (DM) of the roots and shoots of each plant was determined separately to an accuracy of ±1 mg (TP 303; Denver Instruments GmbH, Göttingen, Germany). The ratio of root dry matter to shoot dry matter weight DM_{root}:DM_{shoot}, and the ratio of shoot dry matter weight to fresh shoot weight ratio (DM_{shoot}:FW_{shoot}) were calculated for each treatment. Subsequently, specific root length (SRL) – root length divided by root dry mass (m g^{-1}) [Bergmann et al. 2020], root tissue density (RTD) – root dry mass divided by fresh root volume (g cm^{-3}), and root length ratio (RLR) – root length divided by whole plant dry mass (m g^{-1}) [Ryser 1996] were calculated for each treatment.

Statistical analysis. A factorial arrangement of 24 treatments (3 nitrogen dose levels × 2 levels of NH_4:N_{total} ratios), 4 replicates each, was employed in a randomized complete block design. Residuals of all variables were tested for equality of variances and normality using Brown-Forsythe and Shapiro-Wilk tests, respectively. Differences regarding harvested yield, biomass indicators, root morphology traits, and nitrate/nitrite concentration were tested by three-way ANOVA, using the PC program SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Each significant ANOVA result ($p < 0.05$) was followed by a Holm-Sidak test at $p < 0.05$ as a post-hoc test. Values given throughout the text are means ± SE. Main contributors of diversity regarding root traits, growth parameters, yield, and leaf N concentration under different growing seasons were assessed by principal component analysis (PCA). To produce a graphical evaluation of their relationships the respective heat-map analysis was performed via ClustVis (https://biit.cs.ut.ee/clustvis/, accessed on 14 February 2025) online program package.

RESULTS

The nitrogen (N) supply did not affect root traits. So did the NH_4:N_{total} ratio (Tab. 4). Although root length (RL), root surface area (RSA), and root length ratio (RLR) were slightly higher at the highest N dose (N120) the differences with N60 and N80 were not significant (Tab. 4). The only exception was root tissue density (RTD), where higher N doses were followed by a substantial reduction ($p = 0.044$) in the tissue density (Tab. 4). On the contrary, the planting season significantly affected most of the root traits. The

Table 4. Root length (RL, m), root surface area (RSA, cm^2), average root diameter (AvgD, mm), root volume (RV, cm^3), specific root length (SRL, m g^{-1}), root tissue density (RTD, g cm^{-3}), and root length ratio (RLR, m g^{-1}) of autumn and spring grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions, at the harvesting day (respectively 107 and 68 days after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are noted in bold

Factors		RL	RSA	AvgD	RootV	SRL	RTD	RLR
N_{total} amount	NH_4-N ratio							
60		1190 ±110	178 ±16.1	0.481 ±0.009	2.12 ±0.19	1357.0 ±148	0.455 ±0.03a	62.4 ±6.43
80		1177 ±59	171 ±7.7	0.466 ±0.008	1.99 ±0.09	1951.0 ±363	0.372 ±0.03b	64.9 ±4.81
120		1301 ±54	184 ±8.2	0.451 ±0.004	2.07 ±0.11	1432.4 ±76	0.384 ±0.01b	67.0 ±3.61
	0.4	1206 ±247	185 ±5.8	0.489 ±0.06	2.26 ±0.07	1707.8 ±249	0.406 ±0.02	66.5 ±3.91
	1	1127 ±330	174 ±7.6	0.499 ±0.09	2.13 ±0.09	1452.5 ±105	0.402 ±0.01	63.1 ±4.33
Autumn		1123 ±45	177 ±6.4	0.46 ±0.04b	2.06 ±0.07b	1779.7 ±123	0.373 ±0.01b	82.0 ±3.52a
Spring		1110 ±50	181 ±7.2	0.52 ±0.08a	2.33 ±0.08a	1380.6 ±237	0.435 ±0.02a	47.6 ±2.22b
Autumn								
60	0.4	1307.8 ±132.1	196.0 ±20.2	0.479 ±0.01	2.351 ±0.26	1779.2 ±226.4	0.330 ±0.02	92.8 ±9.1
	1	1073.2 ±176.6	160.0 ±24.6	0.480 ±0.01	1.905 ±0.27	1739.1 ±431.2	0.391 ±0.06	75.0 ±15.1
80	0.4	1235.8 ±62.2	185.3 ±8.79	0.478 ±0.01	2.218 ±0.12	1712.2 ±184.2	0.364 ±0.05	79.2 ±7.5
	1	1118.8 ±100.9	157.8 ±10.5	0.454 ±0.01	1.777 ±0.08	1760.7 ±269.2	0.384 ±0.04	80.6 ±10.5
120	0.4	1241.4 ±49.1	177.0 ±8.34	0.453 ±0.04	2.009 ±0.11	1491.1 ±79.4	0.423 ±0.02	78.9 ±2.6
	1	1361.5 ±95.4	191.2 ±14.4	0.447 ±0.08	2.142 ±0.18	1758.9 ±183.6	0.386 ±0.04	82.1 ±4.8
Spring								
60	0.4	1148.9 ±47.8	175.6 ±9.31	0.486 ±0.01	2.146 ±0.16	926.3 ±111.1	0.626 ±0.07	46.4 ±4.19
	1	789.1 ±105	132.8 ±10.9	0.551 ±0.03	1.812 ±0.13	983.3 ±166.1	0.473 ±0.03	35.2 ±4.28
80	0.4	1124.1 ±128	178.8 ±21.4	0.508 ±0.01	2.280 ±0.31	1712.1 ±90.1	0.364 ±0.04	79.2 ±5.36
	1	1282.3 ±132	201.1 ±19.5	0.502 ±0.01	2.518 ±0.24	1390.0 ±111.2	0.375 ±0.02	52.7 ±5.26
120	0.4	1183.3 ±155	197.9 ±15.8	0.529 ±0.02	2.580 ±0.11	1397.0 ±114.6	0.326 ±0.02	54.2 ±6.37
	1	1136.9 ±94.6	204.0 ±14.9	0.559 ±0.01	2.656 ±0.23	1082.8 ±82.4	0.402 ±0.03	52.7 ±4.61
Significance								
N_{total} dose (A)		0.159	0.069	0.426	0.099	0.157	**0.044**	0.668
NH_4:N_{total} ratio (B)		0.228	0.250	0.307	0.242	0.349	0.884	0.423
Growing season (C)		0.092	0.679	**<0.001**	**0.018**	0.145	**0.033**	**<0.001**
A × B		0.072	0.079	0.110	0.187	0.435	0.578	0.180
A × C		0.298	0.110	**0.025**	**0.030**	0.489	**0.006**	0.297
A × B × C		0.322	0.341	0.587	0.361	0.803	0.068	0.751

Table 5. Dry matter of roots (DM_{root}, g plant^{-1}), dry matter of shoots (DM_{shoot}, g plant^{-1}), fresh weight of shoot (FW shoot, g plant^{-1}), dry matter of roots:dry matter of shoots ratio (DM_{root}:DM_{shoot}), dry matter of shoots:fresh weight of shoots ratio (DM_{shoot}:FW_{shoot}), leaf number (LN, leaves plant^{-1}), leaf area (LA, cm^2 plant^{-1}), and yield (kg variant^{-1}) of autumn and spring grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions, at the harvesting day (respectively 107 and 68 day after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are indicated in bold

Factors									
N_{total} amount	NH_4-N ratio	DM_{root}	DM_{shoot}	FW_{shoot}	DM_{root}:DM_{shoot}	DM_{shoot}:FW_{shoot}	LN	LA	Yield
60		0.709 ±0.04	18.38 ±1.15	8.83 ±0.96b	0.049 ±0.001	0.067 ±0.003	30.5 ±0.58	5602 ±352	8.83 ±0.96b
80		0.688 ±0.06	19.16 ±1.13	9.37 ±0.98a	0.043 ±0.002	0.065 ±0.003	29.7 ±0.58	5839 ±344	9.37 ±0.98a
120		0.827 ±0.05	17.98 ±0.59	9.09 ±0.81ab	0.051 ±0.002	0.064 ±0.003	30.2 ±0.31	5481 ±181	9.09 ±0.81ab
	0.4	0.885 ±0.05	18.76 ±0.84	9.05 ±0.75	0.048 ±0.002	0.066 ±0.003	30.66 ±0.41a	5718 ±263	9.05 ±0.75
	1	0.832 ±0.03	18.25 ±0.75	9.14 ±0.73	0.046 ±0.001	0.065 ±0.003	29.63 ±0.40b	5564 ±229	9.14 ±0.73
Autumn		0.741 ±0.03b	14.39 ±0.28b	5.57 ±0.11b	0.051 ±0.01a	0.082 ±0.001a	28.69 ±0.36b	4385 ±87.7	5.57 ±0.11b
Spring		0.976 ±0.04a	22.62 ±0.51a	12.6 ±0.14a	0.042 ±0.01b	0.048 ±0.001b	31.61 ±0.31a	6896 ±156	12.6 ±0.14a
Autumn									
60	0.4	0.758 ±0.07	13.36 ±0.70b	5.01 ±0.15c	0.056 ±0.002	0.085 ±0.003	28.83 ±0.83b	4074.4 ±214b	5.01 ±0.15c
	1	0.661 ±0.04	14.03 ±0.41b	5.29 ±0.25bc	0.047 ±0.002	0.084 ±0.003	28.83 ±0.70b	4278.1 ±125b	5.29 ±0.25bc
80	0.4	0.703 ±0.12	14.50 ±0.74b	5.54 ±0.22bc	0.048 ±0.007	0.083 ±0.003	28.33 ±1.05b	4420.1 ±225b	5.54 ±0.22bc
	1	0.672 ±0.06	13.82 ±1.14b	5.58 ±0.23bc	0.049 ±0.005	0.079 ±0.002	27.16 ±1.22b	4213.7 ±349b	5.58 ±0.23bc
120	0.4	0.839 ±0.03	14.89 ±0.43b	5.81 ±0.08bc	0.056 ±0.002	0.082 ±0.003	30.00 ±0.57b	4540.6 ±133b	5.81 ±0.08bc
	1	0.814 ±0.09	15.70 ±0.25b	6.17 ±0.081b	0.051 ±0.005	0.081 ±0.003	29.00 ±0.73b	4787.1 ±78b	6.17 ±0.081b
Spring									
60	0.4	1.305 ±0.11	24.16 ±1.71a	448.2 ±11.8a	0.053 ±0.003	0.051 ±0.002	33.66 ±0.88a	7364.8 ±522a	12.55 ±0.33a
	1	0.857 ±0.09	21.95 ±1.72a	446.4 ±6.8a	0.039 ±0.002	0.047 ±0.001	30.83 ±1.10a	6691.9 ±524a	12.50 ±0.19a
80	0.4	0.86 ±0.15	24.83 ±0.69a	480.3 ±11.8a	0.034 ±0.006	0.049 ±0.001	32.16 ±0.16a	7569.6 ±211a	13.20 ±0.21a
	1	0.919 ±0.03	23.48 ±1.00a	469.6 ±8.9a	0.039 ±0.001	0.049 ±0.001	31.16 ±0.60a	7156.3 ±305a	13.15 ±0.25a
120	0.4	0.844 ±0.07	20.80 ±0.48a	436.6 ±9.4a	0.040 ±0.003	0.045 ±0.001	31.00 ±0.36a	6341.1 ±146a	12.22 ±0.26a
	1	1.069 ±0.11	20.53 ±0.47a	433.9 ±19.4a	0.051 ±0.004	0.048 ±0.001	30.83 ±0.40a	6257.2 ±143a	12.15 ±0.54a
Significance									
N_{total} dose (A)		0.189	0.207	**0.015**	0.057	0.323	0.324	0.171	**0.025**
NH_4:N_{total} (B)		0.328	0.355	0.851	0.465	0.514	**0.027**	0.355	0.580
Grow. season (C)		**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
A × B		0.016	0.595	0.758	0.027	0.645	0.751	0.456	0.915
A × C		0.175	0.002	**0.001**	0.540	0.740	0.065	0.012	**0.001**
A × B × C		0.068	0.682	0.994	0.216	0.617	0.219	0.654	0.894

spring-grown lettuce plants had substantially higher average root diameter (AvgD), root volume (RootV), and root tissue density (RTD) than autumn-grown lettuce plants, but significantly smaller root length ratio (RLR). Yet, no differences were found regarding RL, RSA, and specific root length (SRL) due to the planting season (Tab. 4). Although no effects of N supply or NH_4:N ratio were found within each planting season for most root traits, the significant interactions between N supply and planting season regarding AvgD, RootV, and RTD make it difficult to identify the impacts of N supply on these specific parameters.

The amount of nitrogen (N) supplied affected the average shoot fresh weight (FW_{shoot}), and the yield of lettuce plants. A significant increase in FW_{shoot} and total harvested yield followed the rise of N dose from 60 to 80 kg ha^{-1} but the further N dose rise to 120 kg ha^{-1} was not followed by any further increase of either yield or FW_{shoot} (Tab. 4). Importantly noting, the N dose effects were season-dependent. The rise of N supply was followed by an increase in the FW_{shoot} and the yield in the autumn plantings, but not in the spring. Yet significantly higher FW_{shoot} and yield were received in spring plantings than in autumn plantings for the same amount of N supply (Tab. 4).

Neither the N_{total} dose, or NH_4:N_{total} ratio affected the dry matter weight of roots (DM_{root}), the ratio of root and shoot dry matter weight (DM_{root}:DM_{shoot}), the ratio of dry to fresh shoot weight (DM_{shoot}:FW_{shoot}), the number of leaves per plant (LN), and total leaf area per plant (LA). As an exception, the only trait affected by the NH_4:N_{total} ratio was LN. A smaller number of leaves per plant was recorded when the total amount of N supplied was in the NH_4 form (Tab. 5). On the contrary, significant differences were noted in all the above parameters due to the planting season. Spring-grown lettuce plants were characterized by a higher number of leaves per plant and larger plant leaf area than autumn-grown plants. They also had higher DM_{root}, DM_{shoot}, and FW_{shoot}, but significantly lower root-to-shoot-dry matter weight (DM_{root}:DM_{shoot}), and dry-to-fresh shoot weight (DM_{shoot}:FW_{shoot}) – as in Table 5.

In addition to the impacts on plant fresh weight and harvested yield, significant N-dose effects were found regarding the nitrogen use efficiency (NUE) from the lettuce plants. In either autumn or spring plantings, the increase in the N supply was followed by a steady decrease in NUE (Tab. 6). Yet, significant differences exist between the autumn and spring plantings, with the latter almost double the NUE values of autumn plantings. As expected, the increase in the N supply was followed by an increase in NO_3 and NO_2 concentrations in the plant. Remarkably, if no yield increase was found when shifting from N80 to N120, a significant increase was found regarding NO_3 or NO_2 concentration. Again, a strong planting season effect was noticed; significantly smaller NO_3 concentrations were found in spring lettuce plants for each respective N dose.

There was no effect of the NH_4:N ratio in NUE either in autumn or spring plantings (Tab. 6). On the contrary, the NH_4:N ratio significantly affected NO_3 and NO_2 concentrations in lettuce plants at harvest time. The increase in the ratio of N-NH_4 versus total N supplied from 40% to 100% was followed by a significant decrease in NO_3 and NO_2 concentration (Tab. 6). That was common to each N dose, either in autumn or spring plantings. Yet, a significant seasonal effect was strongly present. Significantly smaller NO_3 concentrations were found in spring-grown plants (317 mg kg^{-1}) compared to autumn-grown plants (1244 mg kg^{-1}). In contrast, the opposite results were found regarding the planting season effect on the NO_2 concentrations (4.92 mg kg^{-1} – autumn season *vs*. 10.9 mg kg^{-1} – spring season) at the harvesting time (Tab. 6).

The NO_3 and NO_2 concentrations in the lettuce plants were significantly falling as the fertilizer withholding period (WHP) was extended. Both reached the peak values immediately after the N supply and were gradually reduced (Tab. 7). Moving from day 5 after N supply (WHP 5) to WHP 12, the NO_3 concentration was reduced from nearly 1500 mg L^{-1} to nearly 600 mg L^{-1}. It was further reduced until the day of harvest (DAT 68, WHP 34) to less than 300 mg L^{-1} (Tab. 7). NO_2 concentration has followed the same course, falling from nearly 30 mg L^{-1} by WHP 5 to less than 15 mg L^{-1} by WHP 34 (Tab. 7).

The principal component analyses (PCA) have shown that the variability of lettuce plants in response to N dose, NH_4:N_{total} ratio, and planting season was largely (97%) determined by the first principal component (PC1). LA, SRL, RL, and NO_3 concentration (in diminishing order) show the highest (positive)

Table 6. Nitrogen use efficiency (NUE, kg kg^{-1}), NO_3 concentration (NO_3, mg kg^{-1}), and NO_2 concentration (NO_2, mg kg^{-1}) of autumn and spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions. NO_3 and NO_2 concentrations are measured on the harvesting day (107 and 68 days after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are noted in bold

Factors		NUE	NO_3	NO_2
N_{total}	NH_4:N			
60		736 ±79a	628 ±104c	5.63 ±0.84c
80		585 ±61a	732 ±136b	8.21 ±1.09b
120		378 ±33b	983 ±182a	10.2 ±1.17a
	0.4	564 ±58	820 ±129a	9.03 ±1.04a
	1	569 ±57	742 ±112b	6.98 ±0.78b
Autumn		342 ±16b	1244 ±66a	4.92 ±0.38b
Spring		791 ±46a	317 ±10b	10.9 ±0.76a
		Autumn		
60	0.4	417.4 ±14.7d	1036 ±2.59d	3.36 ±0.017j
	1	441.5 ±14.7d	906 ±3.75e	2.59 ±0.037k
80	0.4	346.7 ±9.51e	1320 ±11.5c	5.54 ±0.066h
	1	349.2 ±9.51e	1027 ±3.46d	5.12 ±0.009i
120	0.4	242.3 ±11.6f	1625 ±4.61a	6.77 ±0.066f
	1	257.1 ±11.6f	1553 ±7.21b	6.22 ±0.063g
		Spring		
60	0.4	1045.8 ±12.4a	282 ±9.16g	9.46 ±0.08d
	1	1041.6 ±12.4a	287 ±7.23g	7.10 ±0.26e
80	0.4	825.0 ±9.41b	290 ±5.81g	13.66 ±0.20b
	1	821.8 ±9.41b	292 ±4.05g	7.50 ±0.28e
120	0.4	509.3 ±3.57c	368 ±1.15f	15.40 ±0.23a
	1	506.2 ±3.57c	387 ±6.17f	12.73 ±0.08c
		Significance		
N_{total} dose (A)		**<0.001**	**<0.001**	**<0.001**
NH_4:N_{total} (B)		0.584	**<0.001**	**<0.001**
Grow. season (C)		**<0.001**	**<0.001**	**<0.001**
A × B		0.903	**<0.001**	**<0.001**
A × C		**<0.001**	**<0.001**	**<0.001**
A × B × C		0.884	**<0.001**	**<0.001**

scores associated with PC1. The remaining traits show uniform (negative) values. Interestingly, having the smallest (negative) value, NUE was also clearly distinguished from the remaining traits. PC2 was responsible for only 3% of variability, with NO_3 concentration, RL, and SRL (in diminishing order) showing the highest (positive) associated scores. Overall, SRL, RL, leaf NO_3 concentration, and NUE appear to be the most important traits that express plant variability (Tab. 8). As such, they can potentially be developed as assisted markers to evaluate the sensitivity of lettuce cultivars to nitrogen supply.

The respective heat map visualizes the differences (Fig. 1). A clear distinction exists between autumn and spring-grown lettuce plants. Within the growing season, a clear separation of N60 with N80 and N120 variants exists in spring-grown plants, with the last two being grouped. On the contrary, the picture is mixed in autumn-grown plants with no clear separation among N60, N80, and N120 variants. Furthermore, the NH_4:N_{total} ratios (0.4, 1) were orderly arranged in the spring-grown but not in autumn-grown lettuce plants (Fig. 1), indicating significant N dose-growing season and NH_4:N_{total} ratio-growing season interactions. Overall, the map confirms that SRL, RL, NO_3 concentration (grouped), and NUE are the most sensitive traits re-

Table 7. NO_3 concentration (mg kg^{-1}), and NO_2 concentration (mg kg^{-1}) of spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions on different fertilizer withholding intervals (WHP; 5, 12, 34) after N application. Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a two-way ANOVA are noticed in bold

N_{total} amount	NH_4-N_{total} ratio	NO_3 concentration (mg kg^{-1})	NO_2 concentration (mg kg^{-1})
		WHP 5	
60	0.4	1474 ±41.3c	27.210 ±0.38b
	1	1274 ±13.4d	25.987 ±0.18b
80	0.4	1532 ±19.6c	26.667 ±0.88b
	1	1218 ±13.5e	25.777 ±0.77b
120	0.4	1860 ±41.7a	35.443 ±0.43a
	1	1604 ±16.2b	33.377 ±0.44a
		WHP 12	
60	0.4	560 ±26.1g	11.653 ±0.20c
	1	307 ±13.5i	11.000 ±0.23c
80	0.4	615 ±9.95g	12.143 ±0.38c
	1	311 ±6.08i	10.467 ±0.29c
120	0.4	729 ±16.2f	13.967 ±0.43c
	1	444 ±11.5h	12.487 ±0.24c
		Harvest day (WHP 34)	
60	0.4	282 ±9.16j	9.467 ±0.08d
	1	287 ±7.23j	7.100 ±0.26d
80	0.4	290 ±5.8j	13.667 ±0.20c
	1	292 ±4.05ij	7.500 ±0.28d
120	0.4	368 ±1.15hi	15.400 ±0.23c
	1	387 ±6.17h	12.733 ±0.08c
		Significance	
N_{total} dose (A)		<0.001	<0.001
NH_4:N_{total} ratio (B)		<0.001	<0.001
Withhold timespan of N application (C)		<0.001	<0.001
A × B		0.044	0.006
A × C		<0.001	<0.001
A × B × C		0.342	0.002

Table 8. The principal components score of root morphology, growth, yield, and fruit quality traits of autumn and spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
DM_{root}	–1.482	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{shoot}	–1.438	–0.181	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{root}:DM_{shoot}	–1.484	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{shoot}:FW_{shoot}	–1.484	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
RL	**1.549**	**0.565**	0.030	0.148	0.165	0.003	0.000	0.000
RSA	–1.021	–0.091	0.000	0.046	0.010	–0.016	0.000	0.000
AvgD	–1.483	–0.179	–0.010	0.014	–0.017	0.001	–0.001	0.000
RootV	–1.478	–0.179	–0.010	0.014	–0.017	0.001	–0.001	0.000
SRL	**2.439**	**1.156**	0.452	–0.036	–0.055	0.000	0.000	0.000
RTD	–1.483	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
RLR	–1.303	–0.109	0.006	0.013	–0.011	0.002	0.012	0.000
LN	–1.407	–0.169	–0.009	0.013	–0.015	–0.001	–0.001	0.003
LA	**12.397**	–0.619	–0.079	0.014	–0.028	0.000	0.000	0.000
Yield	–1.462	–0.184	–0.010	0.015	–0.017	0.001	–0.001	0.000
NUE	**–0.138**	–0.515	0.090	–0.240	0.106	0.000	0.000	0.000
NO_3 concentration	**0.742**	**1.405**	–0.398	–0.090	–0.016	0.000	0.000	0.000
NO_2 concentration	–1.465	–0.183	–0.012	0.019	–0.020	0.001	0.000	–0.001

garding N doses and NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to the growing season.

DISCUSSION

Commonly, an increase in N dose application is followed by a significant enhancement in plant growth [Balliu et al. 2007b, 2009]. However, within the 60 to 120 kg^{-1} ha total nitrogen range, we did not find significant effects regarding lettuce root parameters. Only slightly higher root length (RL), root surface area (RSA), and root length ratio (RLR) values were recorded at the maximum nitrogen (N) supply rate. Within a certain range of N applications, Babaj et al. [2021] have reported similar results for pepper seedlings grown in small containers. The root development is restrained under N supply limitations [Kiba and Krapp 2016]. Hence, since there was no difference in RL and RSA as the N supply was increased, we conclude that even the 60 kg ha^{-1} N supply regime provides enough N for the greenhouse-cultivated lettuce plants. The following discussion on the impact of N dose on the harvested yield confirms that conclusion. Although a common decrease followed the increase in the NH_4:N_{total} ratio from 0.4 to 1.0, no significant effects of the NH_4:N_{total} ratio were found regarding root morphology traits. Similar results were reported by Wang and Shen [2011] that analyzed root length and root surface area of hydroponically grown lettuce within the range of 0.25 to 0.5 NH_4:N_{total} ratio.

The amount of nitrogen supplied did not affect the dry matter of either roots (DM_{root}) or shoots (DM_{shoot}), but a significant effect was noticed regarding the average shoot fresh weight (FW_{shoot}). The increase in N application dose within the range from 60 to 80 kg ha^{-1} led to an increase in the harvested yield, but the further increase to 120 kg ha^{-1} was not followed by any further increase in the yield. By itself, our results are almost identical to a previous report of Thouraya et al. [2022]. They tested the effect of several N dose applications (0, 40, 60, 80, and 120 kg ha^{-1}) in different types of

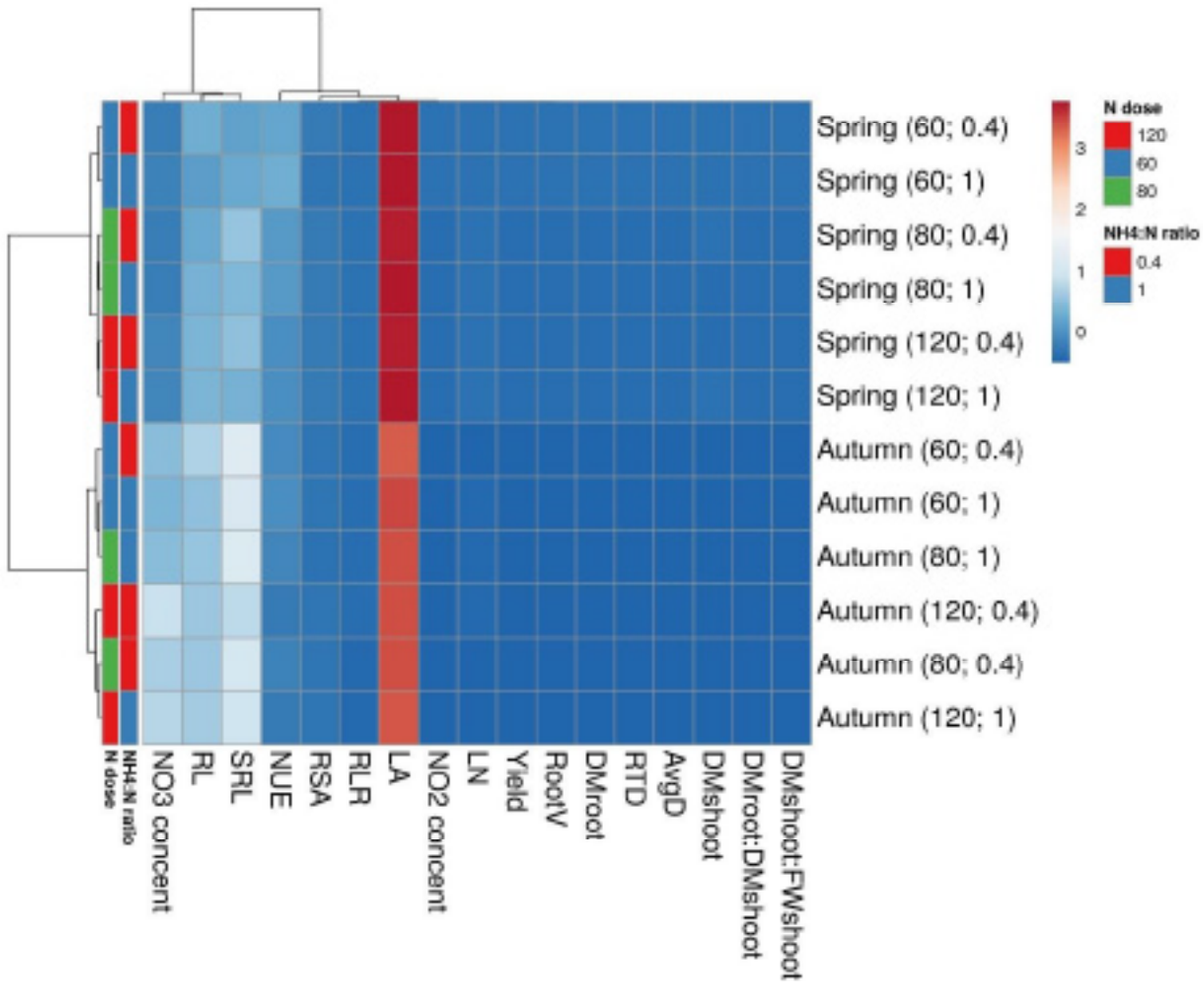

Fig. 1. The heatmap of root morphology, yield, and fruit quality traits of autumn and spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions. Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using correlation distance and average linkage

lettuce and found that the highest yields were obtained in the range from 60 to 80 kg ha^{-1}. The excessive additions of N fertilizers might even reduce the productivity of lettuce crops because of limitations on the K uptake and the reduction of the stomatal conductance [Albornoz and Lieth 2015]. Yet, it is important to note that the differences we found exist only in the autumn season. No differences were found regarding the yield in the spring season concerning N dose applications.

While there was almost no increase in the harvested yield, a significant, steady decrease in nitrogen use efficiency (NUE) followed the increase in the N dose application. It fell from nearly 1000 kg kg^{-1} to 500 kg kg^{-1} in the spring season and from slightly above 400 kg kg^{-1} to almost 250 kg kg^{-1} in the autumn season. Very similar to us, Saah et al. [2022], have reported an increase in lettuce NUE following an increase in N dose application from 31.3 to 62.5 kg ha^{-1} and later a steady decrease following a further increase in N dose application to 93.8 and 125 kg ha^{-1}.

Opposite to NUE, the increase in the N supply was followed by a steady increase in NO_3 and NO_2 concentrations in the plant. Both reached the peak values at the highest N dose applications (120 kg ha^{-1}). Many authors

[Cometti et al. 2011, Urlić et al. 2017a, Thouraya et al. 2022] have reported similar findings. The imbalance between nitrate absorption and reduction, i.e., the plants absorb more nitrates from the soil than is required for their growth, is supposed to be the reason for nitrate accumulation in the plants [Bian et al. 2020].

Different from El-Ghany et al. [2022] who reported higher yields when all N was supplied as NH_4 versus the combined application of NO_3 and NH_4, we did not find any significant effect of the N-NH_4:N_{total} ratio in lettuce yield. Although toxicity symptoms are reported in cases when the total amount of N supplied was in NH_4 form [Guo et al. 2002, Martínez-Moreno et al. 2024], we did not notice any. Apart from the fact that the different crops express different levels of susceptibility, other factors such as rooting medium (soil, soilless substrate, pure nutrient solution) influence the response of plants to the N-NH_4:N_{total} ratio, mostly due to the cation exchange capacity which alters NH_4^+ availability in the root zone and the rhizosphere pH [Savvas et al. 2006]. Natural soil, as in our case, offers higher protection capabilities than soilless production regarding any potential toxicity issues regarding high N-NH_4 dose applications. In addition, we used urea as a source of N-NH_4, which is only gradually converted to NH_4, and the fertilizers were supplied in two doses, avoiding high NH_4 concentrations in the soil that might potentially negatively affect the lettuce plants.

Interestingly, the increase in the N-NH_4:N_{total} ratio from 0.4 to 1.0 was followed by a significant decrease in NO_3 and NO_2 concentration. That was common to each N dose, either in autumn or spring plantings. Similar results were reported by Burns et al. [2011] and Martínez-Moreno et al. [2024] in lettuce and Zhu et al. [2021] in flowering Chinese cabbage. An explanation for this phenomenon is provided by Kronzucker et al. [1999]. According to them, the exposure of plant roots to NH_4^+ imposes a reduction in NO_3^- influx and the enhancement of NO_3^- efflux. The result will be a lower N-NO_3 concentration in the plant tissues. Furthermore, similar to Borgognone et al. [2016] we found that moving from day 5 after N supply (WHP 5) until the harvest day (WHP 34) of spring-grown lettuce, the NO_3 concentration was reduced from nearly 1500 mg L^{-1} to less than 300 mg L^{-1}. The NO_2 concentration followed the same course, falling from nearly 30 mg L^{-1} by WHP 5 to less than 15 mg L^{-1} by WHP 34. Tabaglio et al. [2020] reported a similar trend of decreasing NO_3 concentration in NFT, spring-grown lettuce plants when they withheld fertilization in periods from 2 to 10 days.

The impacts of the growing season were more significant than the N dose application and N-NH_4:N_{total} ratio. It heavily impacted root morphology traits, the dry and fresh plants' weight, N use efficiency, and N concentration in plant tissues. Spring-grown lettuce plants tend to have thicker roots, which leads to a larger root volume and a significantly higher root tissue density. Interestingly, although there were no differences in total root length (RL), the spring-grown lettuce plants had a significantly smaller root length ratio (RLR). Since RLR (m g^{-1}) represents the ratio of root length (RL) to total plant weight, a reduction in RLR indicates a significantly lower investment of photosynthates of the spring-grown lettuce plants towards the root system. That is further supported by a greater root tissue density (RTD), indicating an increased root longevity of spring-grown lettuce plants [Ryser 1996].

The spring-grown lettuce plants showed significantly enhanced nutrient uptake capabilities. Overall, the N use efficiency in spring-grown plants was twice as high as in autumn-grown plants. Was that a consequence of improved environmental conditions, i.e., higher soil and air temperatures [Balliu and Sallaku 2021], improved radiation [Fu et al. 2017], or enhanced plant symbiotic activities with soil microorganisms facilitated by thicker roots? This remains an open question. Not to forget, a significantly larger AvgD of spring-grown lettuce plants might indicate a higher colonization rate from the arbuscular mycorrhizal fungi [Bergmann et al. 2020, Ma et al. 2018] than the autumn-grown plants and potentially higher exudation rates of the root enzymes [Sallaku et al. 2022, Williams et al. 2022]. Both options have significantly boosted the plant's uptake capabilities.

The photosynthates saved in root system construction were invested in the above-ground organs. The spring-grown lettuce has shown significantly lower root-to-shoot dry matter weight (DM_{root}:DM_{shoot}), and lower dry-to-fresh shoot weight (DM_{shoot}:FW_{shoot}) than the autumn-grown plants. In addition, the spring-grown lettuce plants did have a higher $FW_{shoot,}$ a higher number of leaves per plant, and a larger plant leaf area than autumn-grown plants. Finally, there was a higher

yield than autumn-grown lettuce plants for the same amount of N supply. Seasonal effects on lettuce production are not unknown. Similar effects were previously reported by Konstantopoulou et al. [2010], and El-Ghany et al. [2022].

Significantly smaller NO_3 concentrations were found in spring-grown plants than in autumn-grown plants for each respective N dose. Similarly, Savvas et al. [2006] and Konstantopoulou et al. [2010] reported lower N-NO_3 concentrations in autumn-grown lettuce plants compared to the winter-grown plants. Among many factors affecting NO_3 uptake and accumulation in vegetable tissues, N fertilization and light intensity have been identified as the major factors [Santamaria 2006, Bian et al. 2015]. The seasonal differences, particularly solar radiation, which strongly impacts nitrate reductase activity, and temperature, which accelerates both nitrification and plant metabolism [Savvas et al. 2006] enforce the differences between the spring and autumn-grown lettuce plants. A greater accumulation of nitrates was recorded in autumn-winter-grown leafy vegetables due to lower natural radiation values, which led to reduced nitrate reductase activity [Urlić et al. 2017b]. That's why different lettuce nitrate maximum levels (limits) are imposed by European Commission Regulation (EC) No. 563/20027, respectively 4000–4500, and 2500–3500 (mg kg^{-1}) for the autumn-winter (October 1–March 31) and spring-summer (April 1–September 30) period [European Commission 2011]. From the qualitative point of view, the nitrate content in our experiment, in both growing seasons and within all tested nitrogen doses, was quite low and always under the limits imposed by European regulations.

CONCLUSIONS

N-NO_3 concentration and NUE were the most sensitive traits to N dose applications and N-NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to seasonal variation of environmental factors. A range of 60–80 kg ha^{-1} N is recommended for the fertilization of greenhouse-grown lettuce. Further increase of N dose applications does not provide a higher yield. On the other hand, an increase in N dose applications was followed by a significant drop in N use efficiency and a significant increase in N-NO_3 concentration in the plant. The NO_3 concentration in the lettuce leaves can be reduced by increasing the ratio of N-NH_4:N_{total} applied and/or by extending the period of the latest N application before harvesting.

Although the impacts of N dose applications on yield, quality, and leaf NO_3 concentrations are largely studied, the picture is not yet fully completed. The influence of cultivar variation, fertigation method (quantitative *vs*. proportional fertigation), and the impacts of slow-release fertilizers on NUE and leaf NO_3 concentration at various environmental conditions remain open questions.

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EVALUATION OF SUSTAINABLE STRATEGIES FOR GREENHOUSE PEST CONTROL IN CHRYSANTHEMUM AND SWEET PEPPER PRODUCTION

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ABSTRACT

The effectiveness of 16 examples of selected sustainable practices in pest control (i.e. application of plant-derived products, microbial agents or inorganic compounds with expected low environmental impact; simultaneous use of already registered active substances against other harmful organisms, and trap cropping) was tested in the protected cultivation of *Chrysanthemum* × *morifolium* Creamist Golden and *Capsicum annuum* Ożarowska against the two-spotted spider mite (*Tetranychus urticae* Koch.) and thrips (*Frankliniella occidentalis* (Pergande), *Thrips tabaci* Lind.). The study identified *Bacillus subtilis*, common nettle manure, willow bark decoction, oregano and cinnamon essential oils as the most promising solutions for reducing spider mite population. However, in the thrips control, the infusion of Canadian goldenrod root showed a high immediate efficacy that was comparable to the abamectin that was used as a reference product. Further research on these substances is recommended to increase their effectiveness, understand their mode of action against pests and determine the impact on crops.

Keywords: two-spotted spider mite, *Tetranychus urticae*, thrips, protected cropping, natural insecticides, integrated pest management

INTRODUCTION

Recently, trends in horticultural production have aimed at minimising its negative impact on the environment, producing safe and residue-free plant products, as well as reducing or even eliminating the use of chemical pesticides. These endeavours are reflected in the growing emphasis on sustainable approaches to plant protection management, such as organic farming and the integrated pest management (IPM) [Baker et al. 2020, Deguine et al. 2021].

In this context, horticultural crops that are grown under cover (e.g. in greenhouses or polytunnels), due to being susceptible to strong pest pressure, have become an area of particular concern. High, stable temperatures and periodically low air humidity levels, coupled with plants being grown as closely as possible to maximise the limited available space, intensify the incidence of common greenhouse pests. These include various species of aphids, thrips or spider mites [Perdikis et al. 2008, Fatnassi et al. 2015]. Due to the favourable microclimatic conditions, greenhouse pests are able to develop a larger population in a relatively shorter time and to complete more generations than in open field crops. This implies high pesticide use, but also the risk of pests developing resistance to currently authorised active ingredients [Tirello et al. 2012, Mani 2022].

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Excessive use of chemical plant protection products particularly affects protected fruit and vegetable crops, as they are usually consumed fresh [Allen et al. 2015, Mahdavi et al. 2022]. Nonetheless, there is a growing awareness of the potentially negative impact of pesticides, not only on employees in the ornamental horticulture sector, but also on the end consumers of ornamental plants, for example, through the residues of active ingredients in cut flowers [Toumi et al. 2016, Pereira et al. 2021]. Therefore, the need to develop alternative or complementary solutions in pest control is increasingly widely discussed in order to support the gradual shift away from dependence on chemical pesticides in greenhouse horticulture.

In order to identify promising strategies that could support sustainable pest control in protected crops, a search of academic databases (Web of Science, Google Scholar) was performed. The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) – hereafter referred to as TSSM – was selected as the target species due to its relevance as a widespread polyphagous pest of most protected crops [Perdikis et al. 2008]. The search covered publications up to April 2025 and used combinations of the following general keywords: *Tetranychus urticae*, *two-spotted spider mite*, *sustainable pest management*, *protected crops*, *greenhouse*, *natural compounds*, *botanical pesticides*. Moreover, a backward and forward snowballing approach was used to identify additional relevant studies from the reference lists of selected papers. As a result, the five general practices have been selected for testing: (*i.*) application of plant-derived products, e.g. extracts, decoctions, manures or essential oils [Kheradmand et al. 2015, Durán-Lara et al. 2020, Jakubowska et al. 2022]; (*ii.*) use of microbial control agents, including bacteria and fungi [Shinde et al. 2010, Al-Azzazy et al. 2020, Chouikhi et al. 2022]; (*iii.*) use of inorganic compounds, natural or manufactured, but with a low expected environmental impact [Alhewairini and Al-Azzazy 2018, Abdelwines and Ahmed 2024]; (*iv.*) exploiting the insecticidal or acaricidal side-effects of active ingredients already authorised for use against other harmful organisms, e.g. fungicides [Sukhoruchenko et al. 2021]; (*v.*) companion planting [van den Boom et al. 2003].

Sweet peppers and chrysanthemums were selected as test plants for assessing the effectiveness of the investigated pest management strategies. Both species hold a prominent position in the Polish greenhouse horticulture sector. For example, in 2023, the total area of crops cultivated under cover in the spring cycle in Poland was 4451.5 ha, with peppers being the leading crop, accounting for 39% of this area. The harvest of peppers in protected cultivation was estimated at 258.7 thousand t, second only to tomatoes [GUS 2024]. Chrysanthemums also play an important role in Polish ornamental plant production. Moreover, both species are suitable model plants for evaluating the effectiveness of spider mite control, with the additional advantage of being combined within a single greenhouse production cycle during the experiment (chrysanthemums following peppers).

The aim of the present study is to assess the effectiveness of various practices suitable for integrated and/or organic production systems in greenhouse pest control, using the example of the protected cultivation of sweet pepper and chrysanthemum. It was hypothesised that at least one of the practices tested would result in a significant reduction in the pest abundance compared to untreated control.

MATERIALS AND METHODS

The experiment was performed in a greenhouse complex belonging to the National Institute of Horticultural Research in Skierniewice, Poland (51°57'36.9"N, 20°08'59.5"E). Two separate, consecutive trials were conducted and the species tested were sweet peppers (*Capsicum annuum*) Ożarowska (from 24th May to 21st June 2024) and chrysanthemums (*Chrysanthemum* × *morifolium*) Creamist Golden (from 24th July to 19th August 2024). The experimental timeline is presented in Figure 1.

Preparation of plant material. The pepper seedlings were prepared in-house, whilst the rooted chrysanthemum cuttings in growing trays were obtained from an external supplier, the TURSCY Horticultural Farm based in Rzgów, Poland. During the initial growing stage, the young plants were kept in an isolated growing chamber to prevent pest infestation. Two foliar treatments with the fungicide Previcur Energy 840 SL (530 g L^{-1} propamocarb + 310 g L^{-1} fosetyl-Al) were performed; no other plant protection products were used. The plants were transplanted into

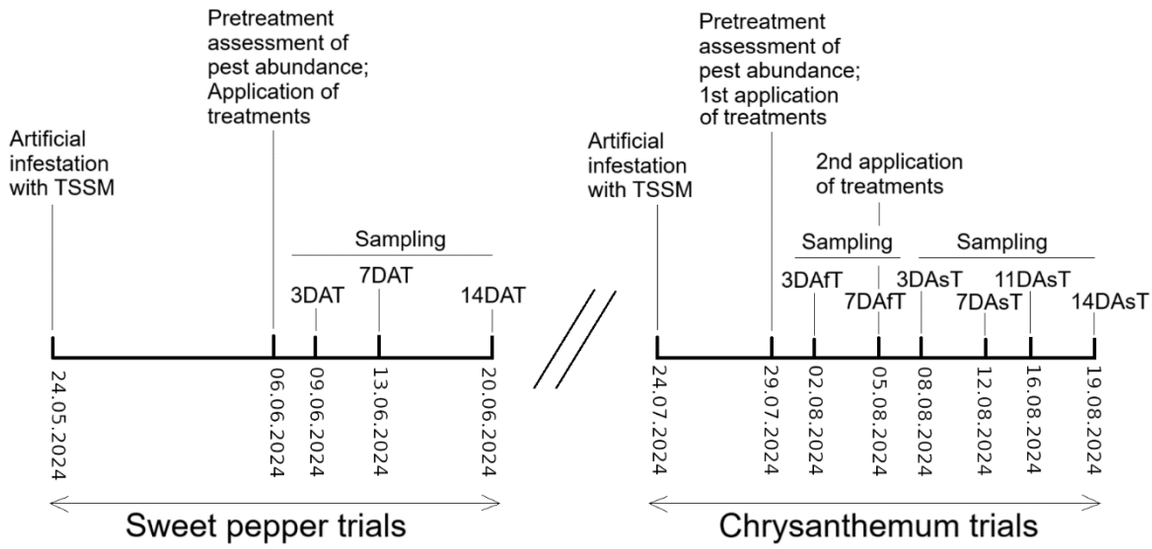

Fig. 1. Timeline of the greenhouse experiments conducted on sweet pepper (from 24th May to 21st June 2024) and chrysanthemum (from 24th July to 19th August 2024), showing the schedule of treatments and assessments. Explanations: TSSM – two-spotted spider mite; DAT – days after treatment; DAfT – days after the first treatment; DAsT – days after the second treatment

1.5 L plastic pots filled with peat-based horticultural medium (H2–H5; pH 6.0–6.5), thoroughly mixed with YaraMila Complex multinutrient fertiliser at a ratio of 1.5 g fertiliser to 1 L medium. Finally, the plants were moved to the target location of the experiment, where they were subjected to pest infestation: the pepper on 24th May 2024 at the stage where the first flower bud was visible (BBCH 51), and the chrysanthemum on 24th July 2024 at the stage before the formation of the flower bud (fewer than 20 true leaves on the main shoot).

Pest breeding and plant infestation. Male and female *T. urticae* mites were collected from an organic strawberry plantation (51°56'46.6"N, 20°11'11.2"E) and bred on common bean plants (*Phaseolus vulgaris*) cv. Eureka in an isolated greenhouse chamber, as described by Byrdy [1965]. Artificial infestation was performed by placing bean leaves with visible feeding symptoms (i.e. with a large but unidentified number of spider mites) on the crops, one leaf per each pepper or chrysanthemum plant. The leaves were not removed afterwards and were left to dry naturally. The incubation period lasted two weeks in the pepper trials and one week for the chrysanthemums, with the differences being due to the higher temperature in July, which favoured the pest's faster development.

In addition, during the pretreatment sampling stage, it was detected that a relatively abundant population of thrips spontaneously occurred in the pepper trials and the decision was taken to include this in the study. A random sample of 30 individuals was therefore collected by shaking them off the leaves and inflorescences. The microscopic preparations were made using the Heinz (PVA) medium. The western flower thrips (*Frankliniella occidentalis* (Pergande)) and the onion thrips (*Thrips tabaci* Lind.) were identified based on the work of Łabanowski [1992]. However, the spontaneous thrips population in the chrysanthemum trials was too scarce and uneven to enable statistical procedures, so the results were excluded.

Trial layout. The methodology was based on the standards of the European and Mediterranean Plant Protection Organisation (EPPO) No PP 1/37(2) and PP 1/160(2) – both modified for the purposes of the present experiment. For both species, each treatment was represented by 24 potted plants, arranged in groups of eight per compartment across three differ-

ent greenhouse chambers (for more information, see Fig. 2). The separate experimental chambers (8 × 4 × 2.8 m) were located in different parts of the greenhouse complex (one in the centre and two at opposite edges). During treatment applications, each plant was sprayed individually. During subsequent maintenance procedures and watering, the potted plants were repositioned within the compartments. Therefore, the assumption was made that each plant was an independent sampling unit, with the greenhouse chamber being regarded as a random effect. During the experiment, the microclimatic conditions were automatically recorded by a sensor system at 30-minute intervals, separately for each greenhouse chamber (Fig. 3).

A total of 16 different treatments were tested across the pepper and chrysanthemum trials, with 10 treatments included in each trial and some overlap between them (Tab. 1). The treatments were compared to water-treated control and abamectin as a reference product. Doses were applied according to the manufacturers' recommendations or, in the case of self-prepared preparations, based on previous laboratory tests (not presented). The tested preparations were dissolved (or suspended) in tap water. For each treatment, approx. 40 mL of liquid per plant were sprayed using a Kwazar Orion manual pressure sprayer. The treatments were carried out late in the evening, at a temperature of approx. 23–24 °C. Careful attention was paid to covering

Fig. 2. The potted pepper and chrysanthemum plants were cultivated on greenhouse growing benches lined with capillary matting. Within a single greenhouse chamber, each treatment was represented by eight potted plants grouped in separate compartments measuring 1 × 1 m. To reduce the risk of pests moving between adjacent compartments, each was separated by an air-permeable fabric with a mesh size of 0.20 × 0.15 mm

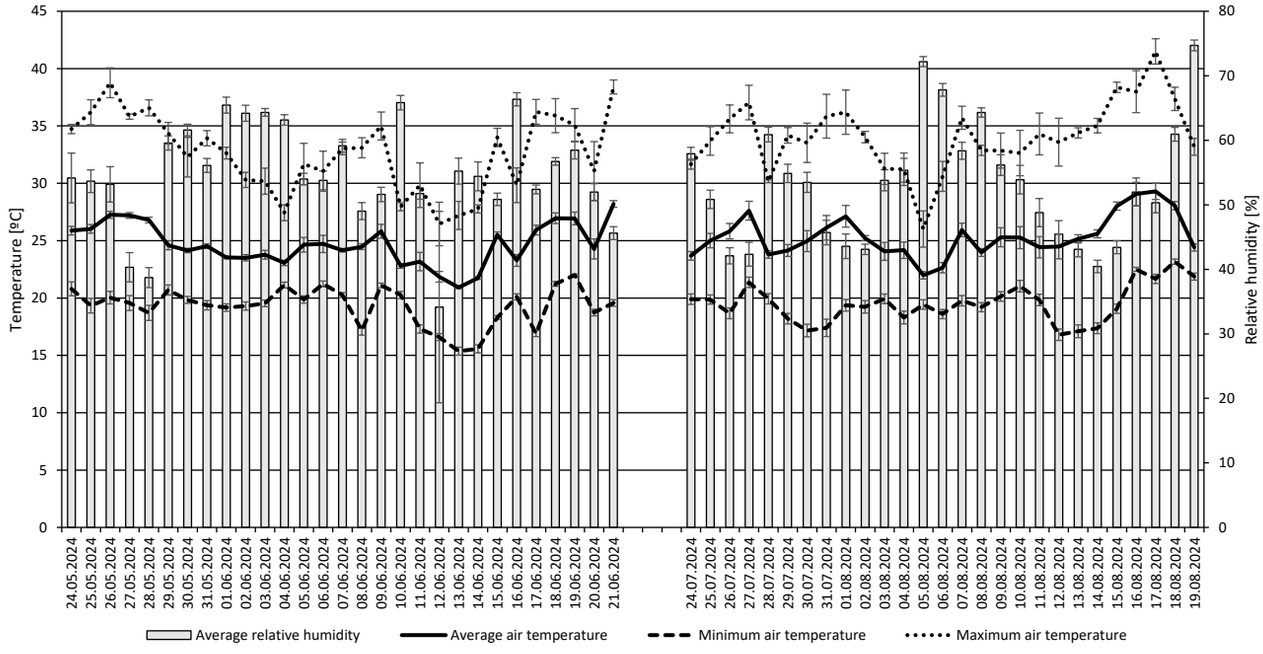

Fig. 3. Temperature and humidity conditions in the experimental greenhouse chambers in which the trials were re-peated. The period from 24.05.2024 to 21.06.2024 covers the experiments conducted on sweet peppers, while the period from 24.07.2024 to 19.08.2024 includes the experiments on chrysanthemums. The values presented are the daily arithmetic means of the particular parameters based on the recordings from all experimental chambers (n = 3), with the standard deviation included

the undersides of the leaves when spraying. Only one treatment was applied to the peppers (6th June 2024), whereas the chrysanthemums were sprayed twice – on 29th July 2024 and one week later. The only exception was the companion planting trial, in which the pepper plants were sprayed with water but placed together with soybeans, with four free-of-pest soybean plants per replicate, in such a way that their leaves were touching. The soybean plants were then removed after one week.

During the experiment, no fertilisers, biostimulants, growth regulators or pesticides (other than those foreseen for the particular treatments) were applied to either the pepper or the chrysanthemum trials. Soil water capacity was maintained at a steady level, with the pots being watered manually 2–3 times per week with approx. 50 mL of tap water. All side shoots were removed by hand as they appeared, leaving only one main shoot. No artificial light was used. The chrysanthemums were not covered with black-out material.

Sampling methodology and pest pressure assessments. At all sampling dates, samples of one leaf from each plant were collected randomly from the middle parts of the shoots. The live mites (including mobile forms – larvae and adults) and eggs, as well as thrips present on the leaves, were counted under the stereoscopic microscope. For both plant species, the initial sampling was performed immediately prior to the first treatment to assess the pretreatment pest pressure (in order to consult the presumptive differences resulting from the artificial TSSM infestation). Moreover, in the pepper trials, the post-treatmnent assessment was performed three times, at 3, 7 and 14 days after treatment (DAT), whilst there were six sampling dates in the chrysanthemum trials: 3 and 7 days after the first treatment (DAfT), and 3, 7, 11 and 14 days after the second treatment (DAsT).

To determine the size of the mite or thrips populations over time, the cumulative index of infestation

Table 1. Specification of treatments applied to control the greenhouse pests

Treatment(s)	Additional information, manufacturer/ method of preparation	Practice tested*	Dose (per 1 L of tap water)	Crop
Abamectin	Safran 018 SC; Rotam Agrochemical Europe Ltd.	Control (reference product)	0.5 mL	Sweet pepper, chrysanthemum
Willow bark decoction	50 g of dried *Salicis cortex* was boiled in 2700 mL of water for 30 minutes, then left to stand for 8 hours, and filtered	*i.*	100 mL	Sweet pepper, chrysanthemum
Infusion of Canadian goldenrod roots	200 g of wet *Solidago canadensis* rhizome was finely chopped, poured with 400 mL of water, then left to stand for 8 hours, and filtered	*i.*	100 mL	Sweet pepper
Tansy extract	Commercially available extract from *Tanacetum vulgare*; BROS Ltd., Poznań, Poland	*i.*	50 mL	Chrysanthemum
Nettle and common wormwood manures	1000 g of wet herb of *Urtica dioica* or *Artemisia absinthium* was poured with 1 L of water and left to ferment in a covered enamelled pot. The content was stirred regularly and water was added as it evaporated	*i.*	100 mL	Sweet pepper
Cinnamon, carnation and oregano essential oils	Water-soluble oil dispersions were obtained from BCHEM Tomasz Miśkiewicz, Rusiec, Poland	*i.*	50 mL	Cinnamon oil – sweet pepper and chrysanthemum; oregano and carnation oils – chrysanthemum only
Biopuls Abracadabra	Commercially available microbial biostimulant containing *Beauveria* spp. and *Metarhizium* spp.; Microlife Ltd., Poznań, Poland	*ii.*	7 mL	Sweet pepper
Serenade ASO	Microbial fungicide containing *Bacillus subtilis* strain QST 713; Bayer AG, Leverkusen, Germany	*ii.*, *iv.*	16 mL	Sweet pepper, chrysanthemum
Akanthomyces lecanii, *Penicillium* sp.	Entomopathogenic fungi isolated from horticultural soil; spore density of *A. lecanii* – 9.7×10^6 per mL, spore density of *Penicillium* sp. – 1.4×10^6 per mL	*ii.*	25 mL	Chrysanthemum
Calcium carbonite	Pure (min. 98%) laboratory $CaCO_3$; WARCHEM Ltd., Zakręt, Poland	*iii.*	50 g	Sweet pepper
Bisteran	Commercially available biostimulant (420–600 g L^{-1} hydrogen peroxide stabilised with silver ions); DESIO HOLDING Ltd., Częstochowa, Poland	*iii.*	0.5 mL	Chrysanthemum
Luna Sensation 500 SC	Commercial fungicide (250 g L^{-1} fluopyram + 250 g L^{-1} trifloxystrobin); Bayer SAS, Lyon, France	*iv.*	1.6 mL	Sweet pepper, chrysanthemum
Companion planting	Soybean plants (*Glycine max*) cv. Erica	*v.*	Not applicable	Sweet pepper

* *i.* – application of plant-derived products; *ii.* – use of microbial control agents; *iii.* – use of inorganic compounds with a low expected environmental impact; *iv.* – exploiting the insecticidal or acaricidal side-effects of active ingredients already authorised for use against other harmful organisms; *v.* – companion planting

(CII) was calculated according to the formula proposed by Wratten et al. [1979]:

$$CII = \sum_{i=1}^{k-1} \left[\frac{t_n}{2} (x_n + x_{n+1}) \right]$$

Where: k is the number of assessments performed; x_n, x_{n+1} are the numbers of mobile mites (or thrips) on two subsequent sampling dates; and t_n is the number of days between the sampling dates.

In addition, the pepper leaves were collected at 14DAT (using the same procedure described above for the pest assessment), scanned at 1200 dpi and subjected to a graphical analysis in the *pliman* programme. The leaf health status, expressed as the ratio of red ('symptomatic') to green ('healthy') pixels, was computed on this basis [Olivoto 2022].

Statistical analysis. The logarithmic transformation $log(x + 1)$ of the data obtained was used to ensure a normal distribution and reach variance homogeneity (except for the CII values, calculated for mobile TSSM on chrysanthemum leaves, which were normally distributed according to the Shapiro-Wilk test).

A one-way analysis of variance (ANOVA) was used to test the significance of differences in the mean values of the pepper leaf health status between treatments.

An analysis of covariance (ANCOVA) was performed to evaluate the effect of treatments on the number of mobile TSSM recorded at different post-treatment sampling points. The response variable was the number of mobile TSSM per leaf at a specific time point. The model included two covariates: the pretreatment number of mobile TSSM and the pretreatment number of TSSM eggs, whilst treatment and greenhouse chamber number were included as fixed factors. The analogous approach was used for the number of TSSM eggs per leaf, with the pretreatment numbers of mobile TSSM and TSSM eggs indicated as covariates in this model, as well as for the number of thrips per leaf, where the pretreatment number of thrips was considered a covariate. In addition, to evaluate the overall effect of the included factors on pest population dynamics, a multivariate analysis of covariance (MANCOVA) was performed with all post-treatment sampling points considered simultaneously as response variables.

An analysis of covariance (ANCOVA) was also used to assess the effect of treatments on the CII values calculated for mobile TSSM and thrips. In these models, treatment and greenhouse chamber number were included as fixed factors, whilst pretreatment counts served as covariates: the pretreatment numbers of mobile TSSM and TSSM eggs were used for the CII of mobile TSSM, and the pretreatment number of thrips was included for the CII of thrips. For these analyses, mean values aggregated at the greenhouse chamber level were used.

Normality (assessed using the Shapiro–Wilk test) and homogeneity of variance (Levene's test) of residuals were confirmed for all the models. The Newman-Keuls test was applied as a post hoc procedure to compare the differences between means. The significance level of $\alpha \leq 0.05$ was adopted for testing the hypotheses. The STATISTICA v.13.3 package was used for all the statistical calculations.

Data availability. The raw experimental data that support the findings of this study are publicly available in the RepOD Repository for Open Data at https://doi.org/10.18150/2WGHRP, reference number 2WGHRP.

RESULTS

Direct effects on the crops. Throughout the observation period, no visible phytotoxic effects (e.g. no chlorosis, stunting, leaf discolouration, deformations, etc.) in response to the pest control methods tested were recorded, in the case of sweet pepper or chrysanthemum plants. A white residue appeared on the pepper leaves and stems after they were sprayed with an aqueous solution of calcium carbonate, however this could be easily wiped off and did not cause any permanent damage.

The above general observations are reflected in the results of the graphical analysis of pepper leaves. The leaf health status did not depend on the type of treatment ($F_{11,251} = 0.90$, $p = .54$, $\eta_p^2 = 0.04$), with the average share of symptomatic leaf area varying slightly between treatments in the range of 0.505–0.588% (detailed results are not presented).

Mobile TSSM on pepper leaves. The mean number of mobile TSSM on the pepper leaves depended significantly on the type of treatment (Wilks' $\lambda = 0.45$, $F_{33,796} = 7.52$, $p < .001$, $\eta_p^2 = 0.24$) and the differing conditions between the greenhouse chambers (Wilks' $\lambda = 0.55$, $F_{6,540} = 31.54$, $p < .001$, $\eta_p^2 = 0.26$). The pretreatment numbers of mobile TSSM (Wilks' $\lambda = 0.98$, $F_{3,270} = 1.51$, $p = .21$, $\eta_p^2 = 0.02$) and TSSM eggs (Wilks' $\lambda = 0.99$, $F_{3,270} = 0.64$, $p = .59$, $\eta_p^2 = 0.01$) explained the variability of this parameter to a negligible extent, with no statistically significant effect.

Common wormwood manure and Serenade ASO significantly reduced the number of spider mites compared to the control at 3DAT, achieving similar efficacy to abamectin (Fig. 4). However, one and two weeks after treatment, only the abamectin application showed a significantly lower number of mobile TSSM compared to the control. The neighbouring soybean plants tended to increase the spider mite pressure on the sweet peppers at all of the sampling dates, although this effect was not confirmed by the statistical test (Fig. 4). Meanwhile, the soybean leaves were severely infested by the pest (an average of 363.0 ±227.4 mobile TSSM per trifoliate leaf on day 10 after the start of the experiment, n = 32).

The CII calculated for mobile TSSM depended significantly on both the treatment type ($F_{11,20} = 2.41$, $p = .04$, $\eta_p^2 = 0.57$) and the number of the experimental chamber ($F_{2,20} = 12.39$, $p < .001$, $\eta_p^2 = 0.55$), with no significant impact of pretreatment numbers of mobile TSSM ($F_{1,20} = 0.07$, $p = .79$, $\eta_p^2 = 0.00$) or TSSM eggs ($F_{1,20} = 0.06$, $p = .81$, $\eta_p^2 = 0.00$). A comparison of the CII values (Tab. 2) shows that only abamectin significantly reduced the spider mite pressure on the pep-

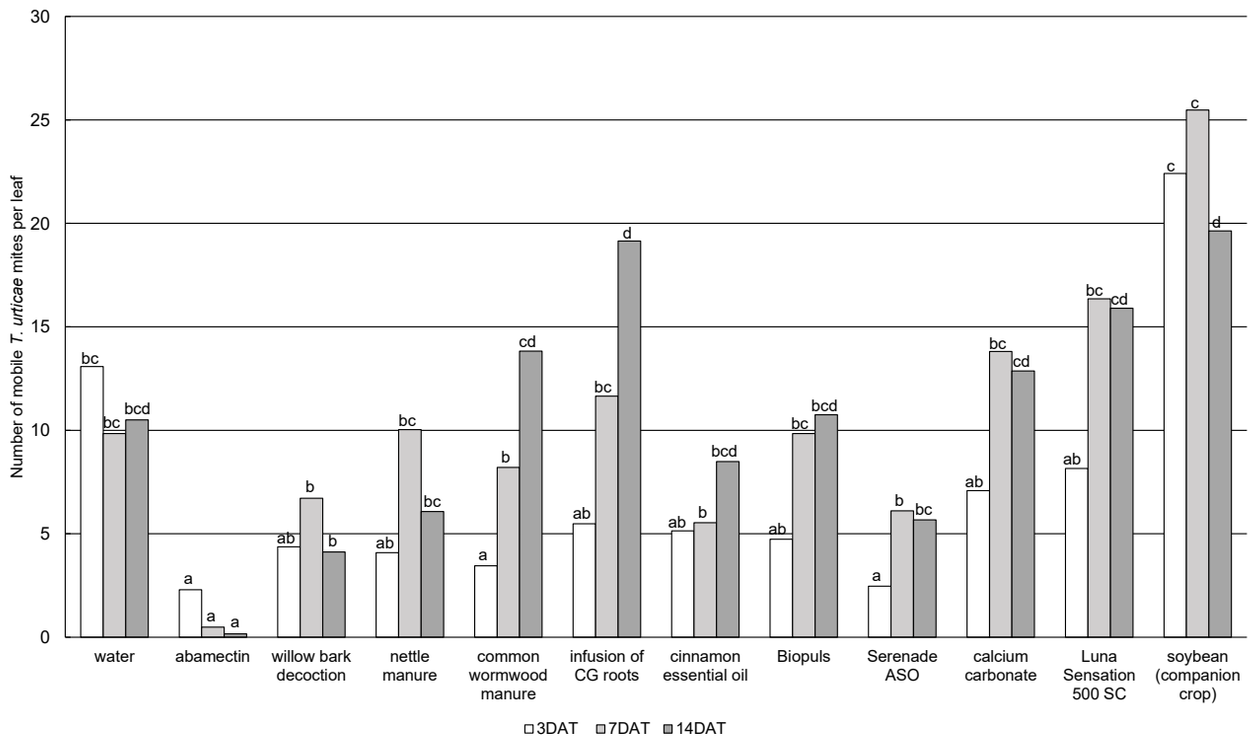

Fig. 4. Effects of different pest control methods on the number of mobile (larvae and adults) two-spotted spider mites on sweet pepper leaves at 3, 7 and 14DAT (days after treatment). Bars represent *(antilog x) – 1* detransformed means (n = 24). Different letters above the bars within each sampling date indicate significant differences at $\alpha = 0.05$, according to the Newman-Keuls test. Explanation: CG – Canadian goldenrod

Table 2. Effects of pest control measures applied on the cumulative index of infestation (CII), calculated for mobile (larvae and adults) two-spotted spider mites on sweet pepper leaves throughout the entire observation period (3–14 days after treatment). The values presented are (*antilog x*) – *1* detransformed means (n = 3)

Treatment	Cumulative index of infestation
Water	1933.3 bc*
Abamectin	111.5 a
Willow bark decoction	997.9 b
Nettle manure	1202.0 b
Common wormwood manure	1589.1 bc
Infusion of Canadian goldenrod roots	2226.8 c
Cinnamon essential oil	1038.3 b
Biopuls	1914.7 bc
Serenade ASO	1053.2 b
Calcium carbonate	1913.5 bc
Luna Sensation 500 SC	2235.1 c
Soybean (companion crop)	3583.3 d

* Means marked with different letter(s) in the column are significantly different at $\alpha = 0.05$, according to the Newman-Keuls test

per leaves, reaching approx. 94% efficacy compared to the control. None of the treatments tested showed efficacy comparable to abamectin over time. An intermediate result in the range of 37.8–48.4% efficacy was obtained after applying willow bark decoction, nettle manure, cinnamon essential oil and Serenade ASO. However, soybean as a companion crop almost doubled the infestation of spider mites in the pepper plants.

TSSM eggs on pepper leaves. The analysis revealed the significant effects of the type of treatment (Wilks' $\lambda = 0.66$, $F_{33,796} = 3.71$, $p < .001$, $\eta_p^2 = 0.13$) and the experimental chamber (Wilks' $\lambda = 0.55$, $F_{6,540} = 31.55$, $p < .001$, $\eta_p^2 = 0.26$) on the number of TSSM eggs on the pepper leaves. The effects of pretreatment numbers of mobile TSSM (Wilks' $\lambda = 0.98$, $F_{3,270} = 1.48$, $p = .22$, $\eta_p^2 = 0.02$) and TSSM eggs (Wilks' $\lambda = 1.00$, $F_{3,270} = 0.19$, $p = .90$, $\eta_p^2 = 0.00$) was not statistically significant.

Although an immediate (3DAT), statistically significant reduction in the abundance of pest eggs was recorded only if calcium carbonate was applied, a downward trend was also observed in plants treated with abamectin, common wormwood manure, infusion of Canadian goldenrod roots, cinnamon essential oil, Biopuls, Serenade ASO and Luna Sensation 500 SC (Fig. 5). One week after treatment, fewer TSSM eggs were counted only on pepper leaves sprayed with abamectin. Nevertheless, a statistically non-significant decrease in response to the application of willow bark decoction, common wormwood manure and cinnamon essential oil should also be noted. Two weeks after treatment, a significant reduction in pest reproduction was observed in both the abamectin and willow bark treatments. However, as with the mobile forms of the pest, the neighbouring soybean plants seemed to favour the mite reproduction at all sampling dates, although this was not reflected in the statistical test results (Fig. 5).

Thrips on pepper leaves. The number of thrips on the pepper leaves depended significantly on both the type of treatment (Wilks' $\lambda = 0.69$, $F_{33,799} = 3.28$, $p < 0.001$, $\eta_p^2 = 0.12$) and the experimental chamber (Wilks' $\lambda = 0.88$, $F_{6,542} = 6.22$, $p < .001$, $\eta_p^2 = 0.06$).

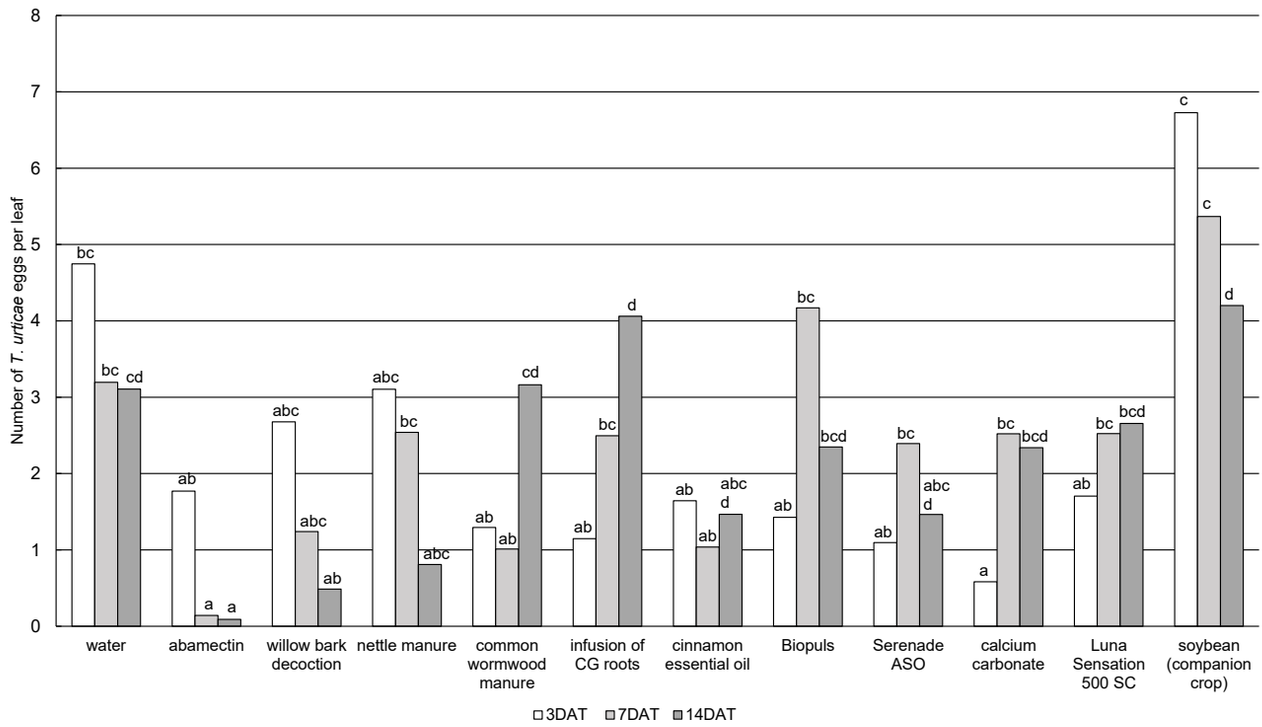

Fig. 5. Effects of different pest control methods on the number of two-spotted spider mite eggs on sweet pepper leaves at 3, 7 and 14DAT (days after treatment). Bars represent *(antilog x) – 1* detransformed means (n = 24). Different letters above the bars within each sampling date indicate significant differences at α = 0.05, according to the Newman-Keuls test. Explanation: CG – Canadian goldenrod

The impact of the pretreatment number of thrips (Wilks' $\lambda = 0.97$, $F_{3,271} = 2.94$, $p = .03$, $\eta_p^2 = 0.03$), although statistically significant, contributed only marginally to the variability of the results. On the other hand, neither the type of treatment ($F_{11,21} = 1.70$, $p = .14$, $\eta_p^2 = 0.47$) nor the experimental chamber ($F_{2,21} = 2.07$, $p = .15$, $\eta_p^2 = 0.17$) significantly affected the CII values computed for thrips (results not presented). This parameter significantly depended only on the pretreatment number of thrips on pepper leaves ($F_{1,21} = 12.02$, $p < .005$, $\eta_p^2 = 0.36$).

As shown in Figure 6, abamectin and infusion of Canadian goldenrod roots reduced significantly the number of thrips on pepper leaves shortly after being applied (3DAT). One week after treatment, only the abamectin-treated plants had fewer pests present, whilst none of the measures provided satisfactory thrips control one week later.

Mobile TSSM on chrysanthemum leaves. The type of treatment (Wilks' $\lambda = 0.43$, $F_{66,1434} = 3.74$, $p < .001$, $\eta_p^2 = 0.15$), the experimental chamber (Wilks' $\lambda = .82$, $F_{12,534} = 4.77$, $p < .001$, $\eta_p^2 = 0.10$), as well as the pretreatment population of mobile mites (Wilks' $\lambda = 0.95$, $F_{6,267} = 2.46$, $p = .02$, $\eta_p^2 = 0.05$) had a significant impact on the number of mobile TSSM on the chrysanthemum leaves although the extent of explained variability was inconsistent.

Significant differences between the control and the substances tested were not observed until 7DAfT, when fewer mobile TSSM were found on the abamectin-treated plants compared to those in the control (Fig. 7). An intermediate result of mite control was recorded after the application of willow bark decoction, tansy extract, oregano essential oil, *Penicillium* sp., Serenade ASO and Bisteran.

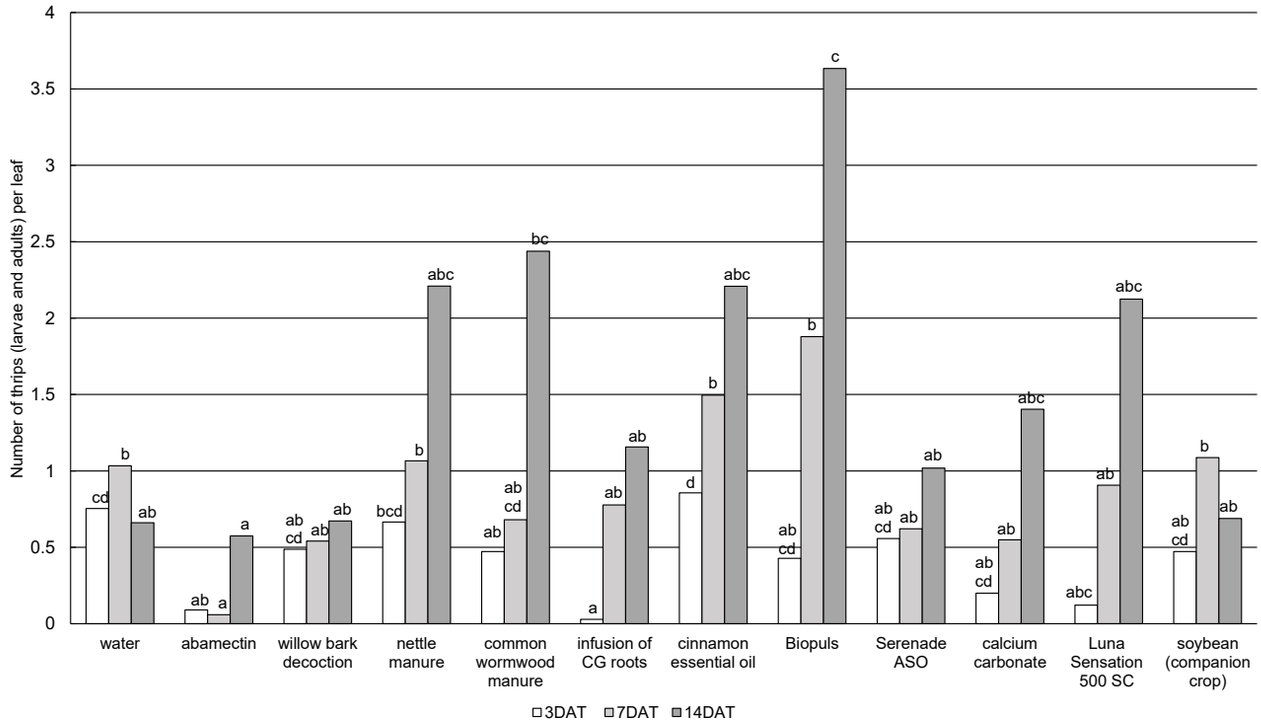

Fig. 6. Effects of different pest control methods on the number of thrips (larvae and adults) on sweet pepper leaves at 3, 7 and 14DAT (days after treatment). Bars represent *(antilog x) – 1* detransformed means (n = 24). Different letters above the bars within each sampling date indicate significant differences at α = 0.05, according to the Newman-Keuls test. Explanation: CG – Canadian goldenrod

In the second series of treatments, the positive effect of the substances tested was solely recorded three days after spraying. At this sampling date, abamectin significantly reduced the number of mobile TSSM compared to the control, whilst the carnation essential oil showed an intermediate efficacy. Later, between 7 and 14DAsT, none of the control methods tested reduced the spider mite pressure on the chrysanthemums; in fact, there was a significant increase in the pest population on plants treated with Luna Sensation 500 SC compared to the control (Fig. 7).

The CII calculated for mobile TSSM present on the chrysanthemum leaves over all sampling dates enables a comparison of the overall response of the pest population to the treatments applied (Tab. 3). The values of this parameter depended significantly on the type of treatment ($F_{11,20} = 2.51$, $p = .04$, $\eta_p^2 = 0.58$), but not on the experimental chamber ($F_{2,20} = 2.99$, $p = .07$, $\eta_p^2 = 0.23$), the pretreatment number of mobile TSSM ($F_{1,20} = 1.87$, $p = .19$, $\eta_p^2 = 0.09$) nor TSSM eggs ($F_{1,20} = 1.34$, $p = .26$, $\eta_p^2 = 0.06$).

According to the data presented in Table 3, abamectin showed the most promising trend towards reducing the pest population compared to the control, with oregano essential oil and Serenade ASO performing less well. Nevertheless, these results were not confirmed by significant differences in the statistical test.

TSSM eggs on chrysanthemums. Although the number of TSSM eggs depended on the type of treatment (Wilks' $\lambda = 0.53$, $F_{66,1134} = 2.71$, $p < .001$, $\eta_p^2 = 0.11$), significant differences between the treatments in the post-hoc comparisons only became apparent at certain time points and generally did not favour any of the approaches (Fig. 8). Other factors, i.e. the experimental chamber (Wilks' $\lambda = 0.88$, $F_{12,534} = 3.01$, $p < .001$, $\eta_p^2 = 0.06$), pretreatment numbers of eggs (Wilks' $\lambda = 0.99$,

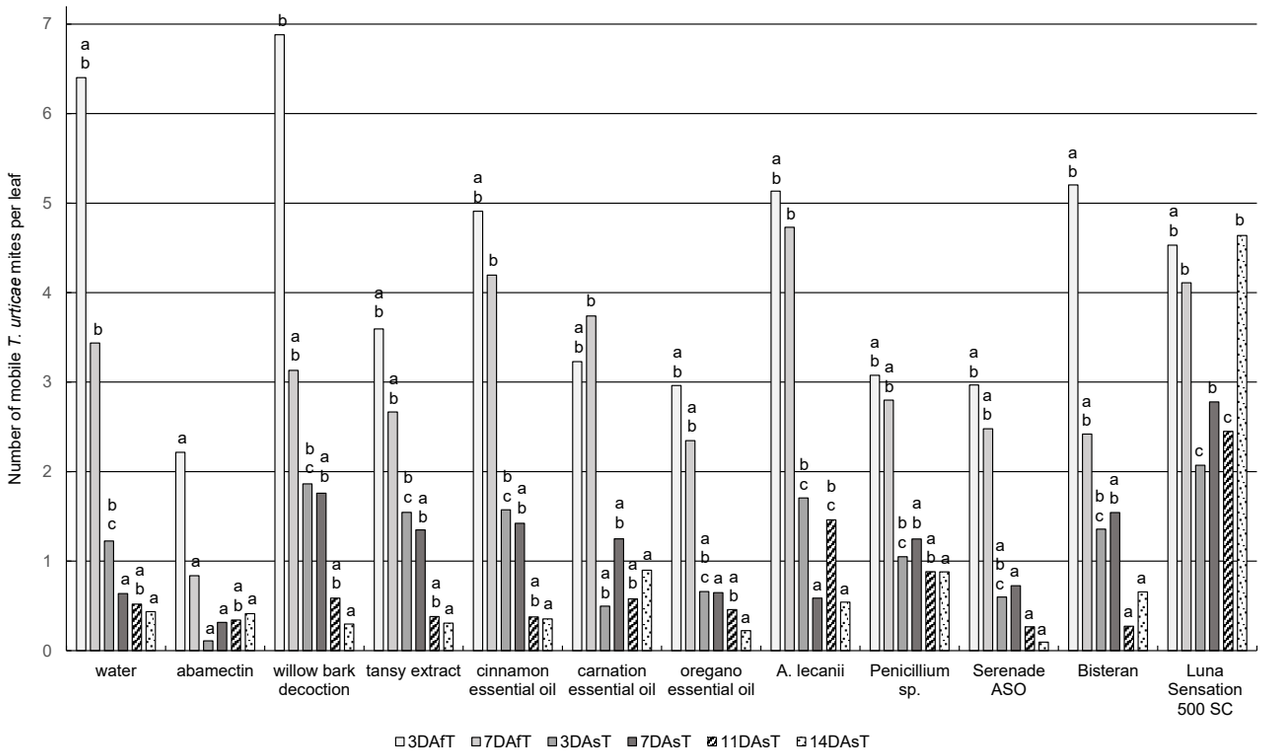

Fig. 7. Effects of different pest control methods on the number of mobile (larvae and adults) two-spotted spider mites on chrysanthemum leaves at 3, 7 DAfT (days after the first treatment) and 3, 7, 11, 14 DAsT (days after the second treatment). Bars represent *(antilog x) – 1* detransformed means (n = 24). Different letters above the bars within each sampling date indicate significant differences at α = 0.05, according to the Newman-Keuls test

$F_{6,267}$ = 0.58, p = .75, η_p^2 = 0.01) and mobile forms (Wilks' λ = 0.99, $F_{6,267}$ = 0.68, p = .67, η_p^2 = 0.01) of TSSM, had even lower or no explanatory power.

DISCUSSION

Sustainable horticultural production is significantly hampered by the high pressure of pests such as TSSM or thrips in protected cultivation. Therefore, there has recently been a strong research focus on the development of alternative pest control methods that could reduce the use of chemical pesticides in greenhouse horticulture. However, it is worth noting that the efforts of researchers have been quite fragmented, with many findings being overlooked and others remaining stuck at the laboratory stage, rather than serving as a stepping stone to future semi-field or full-scale trials [van den Boom et al. 2003, Kheradmand et al. 2015, Abdelwines and Ahmed 2024]. In this context, the present study was intended as a means of collecting and testing various promising ideas with a view to outlining further directions for research on a larger scale, and it has fulfilled its objective.

Application of plant-derived products. This study assessed various plant-derived preparations with expected or previously reported pest control applications. Here, the sole focus will be on those which the results have demonstrated as being potentially useful in plant protection.

Of the essential oils tested, in the pepper trials, cinnamon oil performed relatively well against TSSM (almost 50% reduction in plant infestation) but not against thrips. In the chrysanthemum trials, on the other hand, cinnamon oil was not as effective. Better re-

Table 3. Effect of pest control measures applied on the cumulative index of infestation (CII) calculated for mobile (larvae and adults) two-spotted spider mites on chrysanthemum leaves throughout the entire observation period (3–7 days after the first treatment and 3–14 days after the second treatment). The values presented are arithmetic means ±standard deviation (n = 3)

Treatment	Cumulative index of infestation
Water	444.7 ±96.8 ab*
Abamectin	149.5 ±70.9 a
Willow bark decoction	550.2 ±402.9 ab
Tansy extract	451.7 ±299.8 ab
Cinnamon essential oil	548.8 ±306.8 ab
Carnation essential oil	380.5 ±59.3 ab
Oregano essential oil	327.7 ±170.3 a
A. Lecanii	571.8 ±164.1 ab
Penicillium sp.	466.2 ±310.7 ab
Serenade ASO	317.0 ±270.1 a
Bisteran	459.2 ±213.7 ab
Luna Sensation 500 SC	791.0 ±176.1 b

* Means marked with different letter(s) in the column are significantly different at $\alpha = 0.05$ according to the Newman-Keuls test

sults were obtained by treating the plants with oregano oil, instead. Previous reports on the acaricidal activity of cinnamon oil include successful laboratory tests performed on the carmine spider mite (*Tetranychus cinnabarinus*) [Nasr et al. 2019], and of oregano oil on *T. urticae* [Tabet et al. 2018].

The usefulness of willow bark decoction for pest control is suggested by the approximate halving of the TSSM infestation level in peppers with, a similar downward trend observed for thrips. However, this effect was not observed in the chrysanthemum trials, which could be explained by differences in the response of particular plant species to the use of this substance and/or variation in microclimatic conditions (Fig. 3). Willow bark is currently approved under the EU regulations as a basic substance with fungicidal properties [Deniau et al. 2019], but no information exists on its application in pest control. It has been known that willow bark is a raw material rich in bioactive compounds, in particular phenolic compounds [Piątczak et al. 2020]. The pest control effect observed in this study could have been due to both the direct interactions of the bioactive compounds with arthropods and the induction of natural plant defence processes [Favaro et al. 2019].

Furthermore, the results obtained in this study indicate a certain potential of nettle manure in the TSSM control. According to Dąbrowski and Seredyńska [2007], aqueous nettle extract not only affected the behaviour of *T. urticae*, repelling the pest and exhibiting antifeedant activity, but also caused direct mortality. However, in the current study, the liquid nettle manure was applied, and the different method of processing the herbal raw material (fermentation instead of extraction) may have resulted in a different chemical composition of the final product, also due to metabolites secreted by microorganisms. Thus, determining the chemical profile and microbial content of the liquid manure should be the starting point for further research.

A particularly interesting finding of this work is the high immediate activity of the Canadian goldenrod root infusion in thrips control, comparable to abamectin as a reference product. Canadian goldenrod

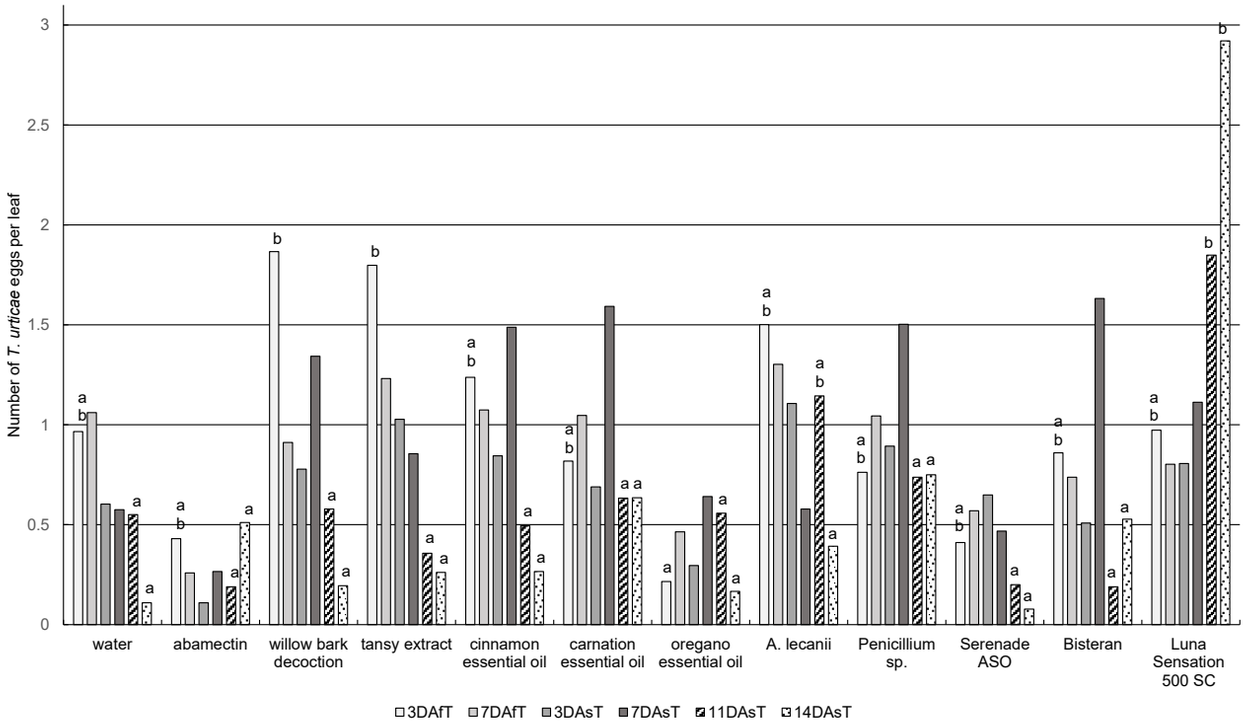

Fig. 8. Effects of different pest control methods on the number of two-spotted spider mite eggs on chrysanthemum leaves at 3, 7 DAfT (days after the first treatment) and 3, 7, 11, 14 DAsT (days after the second treatment). Bars represent (*antilog x*) – *1* detransformed means (n = 24). Different letters above the bars (if present) indicate significant differences at $\alpha = 0.05$ within each sampling date, according to the Newman-Keuls test

is an invasive, fast-spreading plant species in many Eurasian countries. It is difficult to eradicate due to its extensive rhizome system, from which the plants can grow back after the above-ground parts have been damaged [Królak 2021]. To date, only one study has been conducted on the potential use of this species in pest control, which identified the moderately repellent properties of the essential oils extracted from the leaves, stems and inflorescences of the Canadian goldenrod [Baranová et al. 2023]. Here, the idea was to evaluate the roots as a potential material in plant protection, assuming that positive results would contribute to the eradication of this alien species by harvesting (and thus destroying) its rhizomes from natural sites. However, the allelopathic effects of Canadian goldenrod on crops should also be taken into account [Możdżeń et al. 2020]. Further research should therefore focus on assessing the physiological response of crops to the doses of goldenrod preparations used in relation to pest control efficacy, and on possible means of mitigating the negative impact.

Use of microbial control agents. Of the microbial control agents tested in this study, bacterium *B. subtilis* appears to be the most promising solution. Although the performance of the product containing it, Serenade ASO, was less pronounced compared to abamectin as the reference substance, the reduction in the TSSM infestation by almost 50% in pepper trials and 30% in chrysanthemum trials compared to water-treated control should be noted. Other studies also provide evidence of the acaricidal activity of *B. subtilis*, both in laboratory and field trials [Al-Azzazy et al. 2020, Emam 2021, Çelik et al. 2023].

Even though the mode of action of *B. subtilis* against phytophagous mites has not yet been sufficiently explained, Al-Azzazy et al. [2020] suggested

a possible direct bacterium-to-pest infestation, based on disease symptoms occurring in spider mites exposed to *B. subtilis*. An interesting research direction could also be to evaluate the impacts of various substances and toxins secreted by *B. subtilis* on spider mites, as well as to compare the control effectiveness between different bacterial strains [Nagórska et al. 2007]. In this study, *B. subtilis* strain QST 713 was applied in order to test its potential impact on pests as a currently registered biological fungicide. However, it is possible that other strains would have achieved a better result.

The other microbiological agents did not achieve the expected effectiveness in pest control, but the results of this study should not be considered definitive in this regard. The lack of effectiveness could be due to unfavourable microclimatic conditions in the experimental greenhouses (Fig. 3), as adequate humidity and temperature play a crucial role in the activity of entomopathogenic microorganisms [Shipp et al. 2003].

Use of inorganic compounds. Simple inorganic compounds could potentially offer an alternative to synthetic pesticides, whose residues are often detected in crops and cause concern for consumers. The chemical properties of calcium carbonate or hydrogen peroxide suggest they act through direct contact with a pest, e.g. by damaging the cuticle and/or causing water loss, optionally by repelling harmful arthropods. Additional benefits of using these substances may include, for calcium carbonate, increasing the tolerance of plants to heat stress and improving the fruit quality [Patanè et al. 2018, Ramírez-Godoy et al. 2018], while hydrogen peroxide (especially enriched with silver ions) might provide a biostimulant effect and plant disease prevention [Orlikowski et al. 2023].

However, the experiment carried out on peppers within this study, apart from the short-term effect of a decrease in TSSM reproduction soon after treatment, does not confirm the effectiveness of calcium carbonate in mite control, contradicting the findings of Abdelwines and Ahmed [2024]. Also, two treatments with Bisteran (which contains silver-stabilised hydrogen peroxide; it is the current equivalent of the product HuwaSan TR50 on the Polish market, which is approved as biostimulant for spraying directly on plants) proved ineffective in reducing the TSSM population on chrysanthemums. The unsatisfactory efficacy of Bisteran could be explained by its insufficient concentration in the treatments performed. Alhewairini and Al-Azzazy [2018] documented a clear increase in the acaricidal activity of HuwaSan TR50 with increasing concentrations in the range of 0.1–0.4%. In this study, the spray was applied at the concentration permitted on the product label for ornamental plants (0.05%), which is probably too low to control the pest. Therefore, biostimulant treatments with Bisteran at the currently recommended dosage may not be sufficient to ensure a parallel pest control effect.

Insecticidal or acaricidal side-effects of already authorised active ingredients. A promising practice, especially in view of the decreasing availability of registered active substances, could be to plan plant protection treatments in such a way as to exploit the possible insecticidal or acaricidal side-effects of chemical products applied against other non-target harmful organisms. Such a possibility was tested by Sukhoruchenko et al. [2021], who reported the high (almost 100%), albeit delayed up to approx. two weeks after application efficacy of the fungicide Luna Tranquility (125 g L^{-1} fluopyram + 375 g L^{-1} pyrimethanil) in the reduction of the TSSM population. Furthermore, the same study showed the product's satisfactory aphicidal activity on the example of the peach-potato aphid (*Myzus persicae*), and the limited effectiveness in the control of the greenhouse whitefly (*Trialeurodes vaporariorum*). In conclusion, the authors suggested that the toxic effect of the fungicide on the non-target arthropods is related to the activity of fluopyram.

Indeed, according to the FRAC classification, fluopyram belongs to the group of SDHI fungicides (succinate dehydrogenase inhibitors). This enzyme plays an important role in both the citric acid cycle and oxidative phosphorylation. Its dysfunction impairs the process of cellular respiration in mitochondria [Bouillaud 2023]. In addition to its antifungal activity, fluopyram has also been found to act as an effective nematicide (classified to the group of mitochondrial complex II electron transport inhibitors – METI). However, it has been proven not to possess activity against other animals, including insects, oligochaetes and vertebrates, which is explained by amino acid differences compared to nematodes

within SDH complex, known to be important for interaction of fluopyram with the target [Schleker et al. 2022].

In the present study, the effect of another fungicide from the Luna series, Luna Sensation 500 SC was evaluated. The results obtained do not support the hypothesis of either the insecticidal or acaricidal activity of fluopyram. On the contrary, in the chrysanthemum trials, a significant increase in the number of both mobile TSSM and their eggs was found after the second application of the pesticide. The mechanism underlying this observation requires further investigation, as little is yet known about the layered interactions between fungicides and plant pests [Margus et al. 2023].

Companion planting. The dispersal of TSSM depends on its varying preferences for various host plants. In turn, host plants affect its reproductive potential and population growth rate [Greco et al. 2006]. In a study by van den Boom et al. [2003], contrasting differences in acceptance rate were observed between soybean and sweet pepper, with the pest showing a strong preference for the former. It was also noted that, given the opportunity, TSSM migrated from the pepper plants in search of an alternative feeding source.

Considering the growing popularity of trap cropping as a specific form of companion planting [Sarkar et al. 2018], as well as the findings outlined above, this study aimed to assess the development of the TSSM population on pepper plants in the simulated companion planting system with soybean. It was assumed that, once it had infested the peppers, the pest would migrate to the more attractive soybean plants, thus causing less damage to the cash crop. The removal of the soybean plants was intended to simulate potential agronomic operations, such as removing potted trap plants from greenhouses after the expected period of their infestation, or mowing, raking and removing the trap intercrop grown alongside.

Indeed, the pest migrated from the peppers to the expected trap plants and established a far more numerous population; this confirms that soybeans create a more favourable environment for TSSM. However, despite the removal of the trap plants, this did not prevent TSSM from infesting the peppers and increasing its pressure almost twofold, making the assessed practice counterproductive. No such stimulating effect of soybean on the infestation of peppers was still observed in the case of thrips.

CONCLUSIONS

A screening study of several sustainable methods of greenhouse pest control allowed for the selection of the most promising solutions recommended for further research, i.e. microorganisms (*Bacillus subtilis*) and plant-derived products (willow bark decoction, oregano and cinnamon essential oils, common nettle manure, infusion of Canadian goldenrod roots). Future studies should focus on explaining their modes of action, increasing their effectiveness (e.g. by identifying synergists, developing strategies for combined and sequential application, extending the durability in the growing environment) and assessing their impacts on plants at the physiological level.

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BIOLOGY AND EPIDEMIOLOGY OF *Valdensinia heterodoxa* Peyronel IN POLAND

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ABSTRACT

In Poland, the first description of the causal agent of the valdensia leaf blight fungus *Valdensinia heterodoxa* was on highbush blueberry in 2011. Our study identified this pathogen in highbush blueberry plantations and on bilberry plants to describe its biology, molecular characteristics, and disease epidemiology. The 115 fungus isolates yielded were divided into 2 groups based on the colony morphology differences on PDA (potato dextrose agar) medium. For the isolates from each group, morphology and growth dynamics were studied on PDA and WOA (weak oatmeal agar) with added highbush blueberry/bilberry/lily of the valley leaf decoction under different light and temperature conditions. The isolates' colony morphology differences were noted depending on the medium type. The best isolate growth was at 20 °C/19 °C-day, 12 °C-night, with a 12-hour photoperiod. Isolate identification using PCR showed nearly 100% nucleotide sequence similarity to reference isolates. On infected leaves, the release height of conidia was 18 cm. The presented results are novel in Poland and have a broader-than-domestic significance.

Keywords: *Vaccinium corymbosum* L., valdensia leaf blight, fungus morphology, mycelium growth rate, PCR detection and identification

INTRODUCTION

For many years, the highbush blueberry (*Vaccinium corymbosum* L.) was considered to be resistant to pathogen diseases. With the increasing number of plantations and the larger area of cultivation of this plant in Poland, the occurrence of new pathogens infecting the species has been reported more frequently. The results of research conducted by Paduch-Cichal et al. [2011] and Cieślińska [2020] revealed the presence of the following viruses in highbush blueberry plants: Blueberry shoestring virus (BSSV), Peach rosette mosaic virus (PRMV), Blueberry scorch virus (BlScV), Tobacco ringspot virus (TRSV), Blueberry

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red ringspot virus (BRRSV), and Blueberry mosaic associated virus (BlMaV). Various bacterial species from the genus *Pseudomonas* [Kałużna et al. 2013] and *Xanthomonas arboricola* [Kałużna and Pothier 2022] were detected in 2012–2013. Nevertheless, mycoses are the most numerous group of highbush blueberry diseases. The most frequent fungal pathogens of the plant include *Botrytis cinerea* Pers., which is a causal agent of gray mold, *Colletotrichum acutatum* J.H. Simmonds and *C. fioriniae* (Marcelino & Gouli) Pennycook, causing anthracnose in highbush blueberry, and *Godronia cassandrae* Peck., i.e. a cause of shoot blight in this plant species [Zalewska et al. 2007, Szmagara 2009, Meszka and Bielenin 2012, Mirzwa-Mróz et al. 2023].

In 2011, a new species of the fungus *Valdensinia heterodoxa* Peyronel, a causal agent of valdensia leaf blight, was detected in highbush blueberry cv. Bluecrop plants in one of the nurseries in Poland [Dzięcioł et al. 2014]. In the subsequent years, Kukuła et al. [2017] investigated the biology of this pathogen and carried out molecular characterization of 40 *V. heterodoxa* isolates originating from highbush blueberry shrubs growing in commercial plantations or nurseries (cv. Bluegold, cv. Bluecrop) in Mazowieckie Voivodeship and from bilberry plants growing in forests in Pomorskie and Lubelskie Voivodeships.

The available literature from Poland does not provide detailed information on the occurrence and harmful effects of valdensia leaf blight. In other European countries the main recommendation is prophylaxis based on maintenance work and fruit harvesting at a different time than at maximum plant wetting. In the case of the presence of the first symptoms of the disease in commercial cultivation, quick removal and burning infected plants are recommended [Annis and Yarborough 2009, Hildebrand et al. 2016].

The aim of the study was to assess the occurrence of *V. heterodoxa* in highbush blueberry plantations and bilberry plants growing in forests that were not adjacent to the highbush blueberry plantations. Additionally, the study was focused on examination of the dynamics of growth of *V. heterodoxa* isolates on various media, determination of the conditions for the production of conidial spores (conidia) by the fungus and the height at which conidia are released.

MATERIALS AND METHODS

Inspection of plantations

The 5-year study was conducted in 18 commercial highbush blueberry plantations located in Mazowieckie (8 plantations), Łódzkie (5), Podlaskie (3), and Lubelskie (2) Voivodeships. Additionally, the other examined species included trees (*Acer macrophyllum* Pursh., *Betula pubescens* Ehrh., *Corylus avellana* L., *Fagus sylvatica* L., *Quercus robur* L., *Q. petraea* Liebl., *Sorbus aucuparia* L.), shrubs (wild raspberries, blackberries), and perennials (*Convallaria majalis* L., *Oxalis acetosella* L., *Polygonatum multiflorum* (L.) All.) growing in the vicinity or at a certain distance from some plantations. The study was also conducted in mixed forests located at some distance from the highbush blueberry plantations in Pomorskie (3 sites), Lubelskie (2) and Małopolskie (3) Voivodeships.

The plantations were inspected using the route method from June to the end of August. The research material consisting of leaves collected from different plant species with visible symptoms of *V. heterodoxa* infection was placed in a parchment envelope and stored at 4 °C for further analyses. Then, they were examined under a dissecting microscope (SZ11, Olympus, Tokyo, Japan) to find conidia of the pathogen. The conidia were viewed under a BX50 light microscope (Olympus, Tokyo, Japan) and photographed using a DP71 camera (Olympus, Tokyo, Japan).

In total, 100 conidia of *V. heterodoxa* collected from each plant species were measured. The total length (CL) and total width (HW) of each fully developed conidium were measured as in Zhao and Shamoun [2010]. The size of the spores was measured using the CellF program (Olympus, Tokyo, Japan), compatible with the camera and the microscope specified above. ANOVA analysis of variance was carried out, and homogeneous groups were determined with the Tukey test. The analysis was performed in Statistica 13.0.

The pathogen species was identified with the use of available mycological keys [Marcinkowska 2012, Farr and Rossman 2013] and based on the results of studies conducted by Peyronel [1923], Redhead and Perrin [1972a, 1972b], and Nekoduka et al. [2012].

Selected conidial structures were observed under a scanning electron microscope (FEI Quanta 200 ESEM

with an EDS EDAX analyzer). Sections with a size of approx. 3 × 3–4 mm were cut from conidia-bearing leaves and placed on a table covered with activated carbon tape. The preparation was then silver-coated in a vacuum sputter coater (JEOL JFC-1300).

Acquisition of *V. heterodoxa* isolates

Leaves with disease symptoms collected during the inspection were processed with two methods to obtain *V. heterodoxa* isolates.

Method I – sections (approx. 5 × 5 mm) were cut from the border between healthy and diseased tissue in the disease spots and disinfected in 96% ethyl alcohol and 10% sodium hypochlorite. Afterwards, they were transferred to Petri dishes (Ø 100 mm) with PDA medium (potato dextrose agar) [Gams et al. 1987], and incubated at 20 °C in daylight. Single-spore cultures were obtained (from the tips of single hyphae in the case of non-sporulating cultures) and transplanted into tubes onto PDA medium slants. The fungal collection was stored at 4 °C.

Method II – single-spore cultures were obtained from conidia that were actively released from the infected leaves [Redhead and Perrin 1972a, 1972b].

Testing Koch's postulates. Approximately 20 cm long leafy shoots were collected from healthy plants of highbush blueberry cv. Bluecrop and bilberry. Young leaves of these shoots (the second and third fully developed leaves; in total 6 leaves per isolate) were inoculated. Isolates differing in the colony morphology on the PDA medium slants were selected for the analyses. In the case of isolates with similar morphology, 10 isolates were selected randomly. After inoculation the plants were tightly closed in transparent polyethylene bags, and incubated at 20 °C. The pathogen was re-isolated from the forming spots, and the re-isolates were compared with the isolates used in the study.

Identification of the causal agent of valdensia leaf blight using the PCR technique

The material consisted of 14-day cultures of 115 isolates of the fungus *V. heterodoxa* and 2 reference isolates: HBI0401 from leaves of highbush blueberry cv. Jersey, Japan (MAFF No. 645023, NIAS – National Institute of Agrobiological Sciences, Genebank Japan) and DAOMC 186993 from leaves of *Gaultheria shallon* Pursh, Canada (Canadian Collection of Fungal Cultures, Biodiversity and Collections).

DNA isolation. Genomic DNA was isolated from 115 isolates obtained from the fungus *V. heterodoxa* parasitizing highbush blueberries and blackberries with the use of the C-tab procedure (cetyltrimethylammonium bromide) described by Doyle and Doyle [1987].

PCR technique. The pathogen species was identified with the use of the PCR technique. Amplification of non-coding rDNA fragments (ITS1 and ITS2) was carried out using primers ITS1F [Gardes and Bruns 1993] and ITS4A [Larena et al. 1999]. The reactions were carried out as described by Nekoduka et al. [2012] with some modifications. The primer annealing temperature was 59 °C instead of 57 °C, and 28 cycles were run instead of 30. The PCR reaction was carried out using a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, USA).

Amplified fragments were separated electrophoretically in 1.2% agarose/TBE gels in the presence of ethidium bromide. The amplified fungal DNA fragments were sequenced in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. The sequences were analyzed and aligned to the sequences of this fungus available in GenBank using the BLASTn algorithm [https://blast.ncbi.nlm.nih.gov/Blast.cgi] and MEGA12 software.

Characterization of *V. heterodoxa* isolates with classical methods

Effect of temperature and light on the growth of selected *V. heterodoxa* colonies. Based on the morphology of the cultures grown on PDA medium, 115 fungal isolates were preliminarily divided into groups. Further analyses were performed using isolates VhVal3A, VhVal4, and VhBg/1z/16 from highbush blueberry and isolates VhDM1, VhKir1, VhK26/2a from bilberry, which differed in the morphology and origin (northern, central, and southern Poland). Reference isolates HBI0401 and DAOMC 18699 were also included in the study.

The inoculum consisted of 5 mm diameter disks of PDA medium overgrown by the mycelium of the analyzed isolates and cut out with a cork borer from actively growing 14-day cultures of each isolate. The

disks were transferred to the center of sterile PDA poured into 100 mm diameter Petri dishes (10 dishes per each isolate). The dishes were incubated in a growth chamber (Versatile Environmental Test Chamber, Sanyo, Japan) at temperatures of 10, 15, 20, and 25 °C in two variants: I) 12h day/12h night, II) no access to light [Nekoduka et al. 2012].

The diameters of the colonies of fungal isolates were measured 28 days after the inoculation. The results were used to calculate the surface area of the colony using the area of ellipse formula:

$$S = \Pi/4 \cdot d \cdot s$$

where: d – colony length, s – colony width.

The experiment was conducted in duplicate. One-way analysis of variance (ANOVA) was performed. The Tukey test (p = 0.05) was used to compare the means. Statistical calculations were made using the Statistica 13.0 program.

Morphology and growth dynamics of selected *V. heterodoxa* isolates cultured on different media and in different incubation conditions

The mentioned above isolates from the highbush blueberry, bilberry, and reference isolates were selected for the morphology studies. The growth dynamic of the fungal colonies was analyzed only in the case of isolates VhVal3A and VhVal4 representing different groups. The morphology and growth dynamics of selected *V. heterodoxa* isolates were analyzed using PDA and WOA (weak oatmeal agar) media supplemented with decoctions of powdered or fresh leaves from selected host plants (highbush blueberry, bilberry, and lily of the valley) placed in Petri dishes (Ø 100 mm).

The media were inoculated using disks overgrown by the mycelial hyphae of the individual isolates as described above. After the inoculation, the Petri dishes were incubated in a growth chamber (Versatile Environmental Test Chamber, Sanyo, Japan) in the following conditions:

– temperature of 19 °C, no light access [Zhao and Shamoun 2006]

– room temperature (20 °C), daylight [Nekoduka et al. 2012]

– temperature of 19/12 °C (day/night), 12-h photoperiod [Vogelgsang and Shamoun 2002].

The morphological features of the pathogen cultures were studied twice on five Petri dishes (Ø 100 mm) per each isolate using the procedure described by Zhao and Shamoun [2006]. The morphology of the fungus colonies was observed four times at 7-day intervals.

In the experiment on the growth dynamics of the *V. heterodoxa* isolates, 10 Petri dishes with each medium were used for each isolate. After 5, 10, and 15 days, measurements of colony diameter were made and the surface area calculated. The rate of the increase in the surface area of the fungal colonies was determined for each of the medium variants with the use of the regression equation:

$$y = a + bx$$

where: y – surface area of mycelium; a – intercept; b – coefficient of the slope of the regression line; x – day of experiment [Krysicki et al. 1994].

Pearson correlation coefficients (r) were calculated. One-way analysis of variance (ANOVA) of the calculated regression coefficients was performed. Homogeneous groups were determined using the Tukey test at the level of p = 0.05. All the statistical calculations were performed using the Statistica 13.0 program.

Formation of *V. heterodoxa* conidia in *in vitro* conditions. The study was conducted using the same set of isolates as mentioned above. The WOA and OA (oatmeal agar) media were inoculated with mycelium-overgrown disks as described above. The experiment was conducted in three variants:

Variant 1. Inoculation of WOA medium on Petri dishes, incubation in a growth chamber, temp. 16 °C, photoperiod 12 h day/12 h night [Nekoduka et al. 2012]

Variant 2. Inoculation of WOA and OA media, incubation in a growth chamber at 17–19/12 °C (day/night), 12 h photoperiod [Vogelgsang and Shamoun 2002, Zhao and Shamoun 2010]

Variant 3. Inoculation of OA medium on Petri dishes, incubation in daylight, temp. approx. 20 °C – modification of conditions developed by the authors of the present study.

Cultures of *V. heterodoxa* isolates growing on the media were observed to detect spore formation on days 7, 14, 21, and 28 after inoculation. The experiments with each of the variants were carried out twice. Three Petri dishes with each medium were allocated for each isolate. The number of conidia of the pathogen formed per day was assessed. The cultures were observed under a SZX16 stereoscopic microscope (Olympus, Tokyo, Japan). The conidia were viewed under a BX50 light microscope (Olympus Tokyo, Japan) and photographed (DP71 camera). The Cell F computer program was used to measure their length and width.

Upon determination of the optimal conditions for the selected isolates to produce spores, the spore formation ability of the other 115 *V. heterodoxa* isolates was analyzed.

Release of *V. heterodoxa* conidia at different heights

The study of the height to which *V. heterodoxa* conidia were released was carried out twice using 2000 ml cylinders. Fresh highbush blueberry and bilberry leaves with visible disease symptoms were rinsed under running water for 2 hours. Next, three leaves of each species were placed on Petri dishes (Ø 50 mm) covered with sterile moistened filter paper. Each dish was covered with a lid with an aluminum cap glued on its upper side. This helped to connect the Petri dish to a threaded rod. The dish with the rod was gently placed on the bottom of a measuring cylinder with the leaves on its underside, and the lid was moved away from the leaf surface at a distance of 5, 10, 15, 20, and 25 cm using the threaded rod as in Redhead and Perrin [1972a, 1972b]. The observations of the Petri dishes placed at the different heights were initiated when conidia produced in the control conditions were visible on the inner surface of the dish lid. A stereoscopic microscope SZ11 (Olympus Tokyo, Japan) was used for the observations.

RESULTS

Occurrence of the pathogen

During our five-year inspection of 18 highbush blueberry plantations and 8 natural stands, the symptoms of valdensia leaf blight were observed several times. In Mazowieckie Voivodeship, the pathogen was noted on highbush blueberry cv. Bluegold and two unknown cultivars (Prażmów plantation). No valdensia leaf blight symptoms caused by *V. heterodoxa* were found in highbush blueberries in the other 17 plantations. In the natural stands, the pathogen was commonly observed each year on wild-growing bilberry shrubs in forests in Kopalino, Karwia, Ostrowo (Pomorskie Voivodeship), Włodawa, Okuninka (Lubelskie Voivodeship), and Zakopane, Witów, Kiry (Małopolskie Voivodeship) and on lingonberry plants growing in the forest in Witów (Małopolskie Voivodeship). The same symptoms were present on blueberry and lingonberry. The disease symptoms were round or oval, brown or almost black circular zonated necrosis outlined by dark-brown borders. On the lower side of the leaves, large star-shaped conidia were observed in the central part of each spot. Additionally, in the material from the forest in Karwia, characteristic disease spots with conidium were detected on the shoots of a young bilberry plant (Fig. 1).

Identification of the pathogen with traditional methods

During our research, 115 *V. heterodoxa* isolates were obtained, including 20 isolates originating from the leaves of highbush blueberry cv. Bluegold, 15 isolates from unknown highbush blueberry cultivars, and 80 isolates from bilberry shrubs. Koch's postulates for the isolates were positive.

Conidia (macroconidia) of *V. heterodoxa* prepared from the central part of the spots had the shape of a four-pointed and, less often, three-pointed star. This type of conidia is called staurospores. They were large and visible with the naked eye in the central part of the spot on the highbush blueberry and bilberry leaves. The conidia measured in the lingonberry material had smallest length and differed significantly from the length of conidia isolated from the other highbush blueberry shrubs. The conidia isolated from the lingonberry leaves had the smallest width and differed significantly from those measured in the bilberry and highbush blueberry samples (Tab. 1).

The conidia of the fungus present in the central part of the spots on the highbush blueberry, bilberry, and lingonberry leaves had "arms" mostly raised upwards. In turn, the "arms" of conidia located in other areas of the spots were spread out (the conidia resembled

Fig. 1. Bilberry shoot with a visible spot with conidium of *V. heterodoxa* (arrow)

Table 1. Comparison of the conidia size of the *V. heterodoxa* spores isolated from different plant species

Plant species	Length of the arms (µm)		Width of the head (µm)	
	min.–max.	average	min.–max.	average
Lingonberry	205.25–332.01	291.21a*	91.5–152.36	118.38a
Bilberry	187.48–391.25	300.71ab	86.33–172.67	129.59b
Highbush blueberry cv. Bluegold	281.28–365.73	310.75b	105.04–184.00	135.91bc
Highbush blueberry cv. unknown 1	216.85–392.31	313.24b	101.99–198.80	138.21c
Highbush blueberry cv. unknown 2	227.46–384.63	312.30b	105.90–188.45	139.35c

* Letters indicate significant (P = 0.05) differences among means within each column, determined using Tukey test

a star). The macroconidia were formed on short conidiophores and represented the so-called "sessile spores". In the central part of each spore, 60 spherical cells were visible. The cells in the further part were elongated and extended, forming four arms. Younger conidia had strongly hook-shaped arms, while the arms of older conidia were spread more extensively (Fig. 2).

PCR-based identification and characterization of the causal agent of valdensia leaf blight in highbush blueberry

The sequencing of ITS region amplicons yielded 101 sequences of the analyzed isolates with a length of 559 nucleotides (nt) and 14 sequences with a length of 558 nt. The consensus sequences were deposited in the GenBank under numbers PV147766 (559 nt sequence,

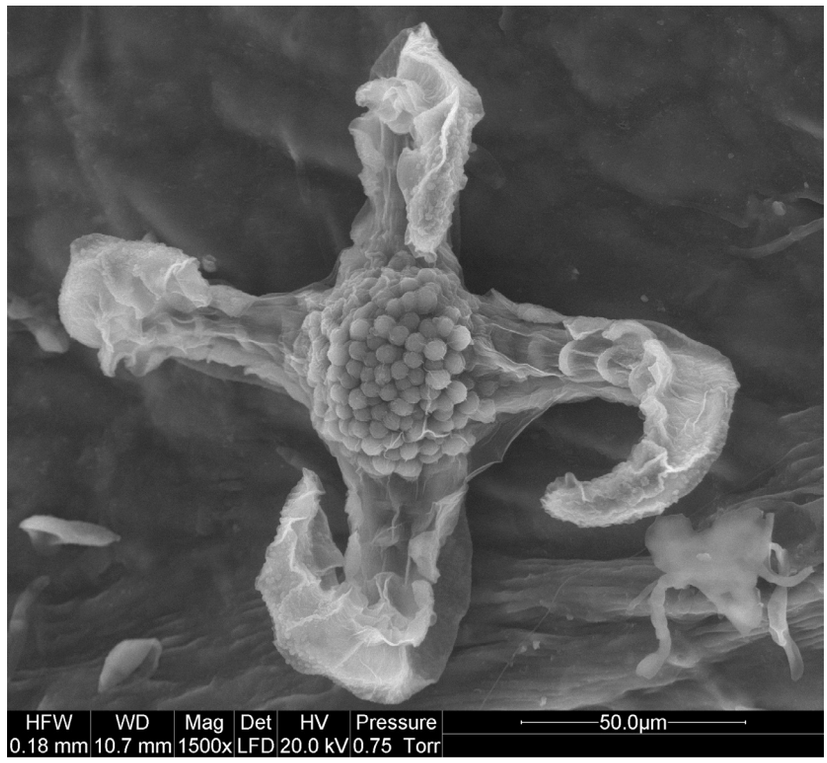

Fig. 2. A macroconidium of *V. heterodoxa* visible under a scanning electron microscope

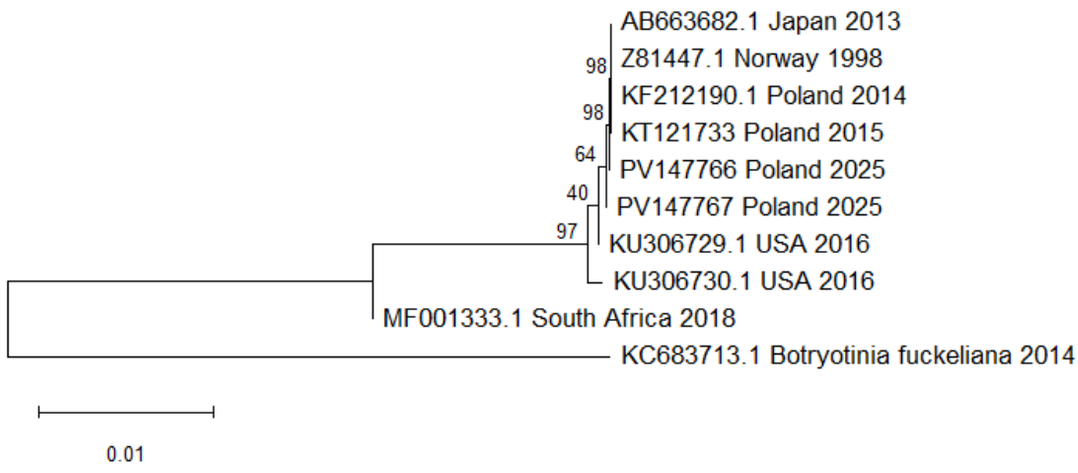

Fig. 3. Comparison of the ITS (ITS1, 5.8 SRNA and ITS2 complete sequence) sequences of the *V. heterodoxa* fungus with the fungal ITS sequences included in the GenBank (Neighbor-Joining method, bootstrap = 1000); the evolutionary distances were computed using the Maximum Composite Likelihood method; low bootstrap support (40%). Tree rooted with the sequence of the *Botryotinia fuckeliana* (KC683713.1) fungus

isolate VhVal3A) and PV147767 (558 nt sequence, isolate VhOstr.2). The differences in the sequences of the isolates from Poland resulted from only a single deletion in the sequences.

These sequences were aligned with very high similarity (99–100%) to the nucleotide sequences of the *V. heterodoxa* species deposited in the GenBank under numbers Z81447.1, AB663682.1, KF212190.1, KT121733 and KU306730.1. The comparison of the ITS sequences of the fungus *V. heterodoxa* obtained in the present study with the sequences of fungi available in the GenBank is presented in the dendrogram (Fig. 3).

Characteristics of *V. heterodoxa* isolates obtained with classical methods

Effect of temperature and light on the growth of selected *V. heterodoxa* cultures. The cultures of tested isolates exhibited the highest growth rate at 20 °C with the 12-hour photoperiod. The statistical analysis showed a significant effect of this temperature on the growth of most of the isolates tested (Tab. 2).

Morphology and growth dynamics of selected *V. heterodoxa* isolates on different media and in different incubation conditions. Given the differences in the morphology of the colonies of the isolates on PDA, the isolates were divided into two groups. The first group comprised 55 isolates whose mycelium surface was corrugated, and some part of the leathery and velvety colony was raised above the medium surface. The color of the upper part of the colony was uniformly dark brown, light cream or speckled. The reverse side of the colony was dark brown or black. The second group comprised 60 isolates producing light cream mycelium with a pink and grayish shade. In the central part, the velvety and leathery mycelium of these isolates was strongly corrugated. The reverse side of the isolates from this group was cream colored. The reference isolates also differed in the appearance of their mycelium. The morphology of the culture of the HBI0401 reference isolate (Japan) was typical of group I isolates, while the morphology of the DAOMC186993 reference isolate (Canada) was characteristic of group II.

The selected fungal isolates, i.e. VhVal3A and VhVal4, as well as VhKir1 and VhK26/2a, grew most efficiently on the PDA medium enriched with highbush blueberry leaves (PDA-Bor) at daylight and 20 °C ambient temperature. The mycelium of both isolates was very well developed, covering practically the entire available surface of the medium in the Petri dishes. The morphology of the fungal colonies differed on the individual plates, depending on the supplementation of the medium (Fig. 4).

Table 2. Average sizes (mm^2) of the cultures incubated under different temperature conditions on the PDA medium

Isolate	Incubation temperature			
	10 °C	15 °C	20 °C	25 °C
VhVal3A	611.20aA*	2984.57bA	6158.17dA	5735.69cB
VhVal4	648.88aA	2369.68bA	6973.47dB	6258.02cC
VhBg/1z/16	695.20aA	3620.34bB	6935.16dB	6258.81cC
VhDM1	796.46aAB	3184.67bA	6125.98cA	5779.64cB
VhKir1	923.16aB	2826.55bA	6954.39dB	5042.13cAB
VhK26/2a	987.84aB	2948.77bA	6373.73dAB	4176.44cA
HBI0401	1144.61aC	3500.32bB	7039.33dB	4863.08cA
DAOMC	1078.59aC	3491.68bB	6302.29dAB	5026.04cAB

* Homogeneous groups according to Tukey test, p = 0.05
Values in rows marked with the same lowercase letter do not differ significantly
Values in columns marked with the same capital letter do not differ significantly

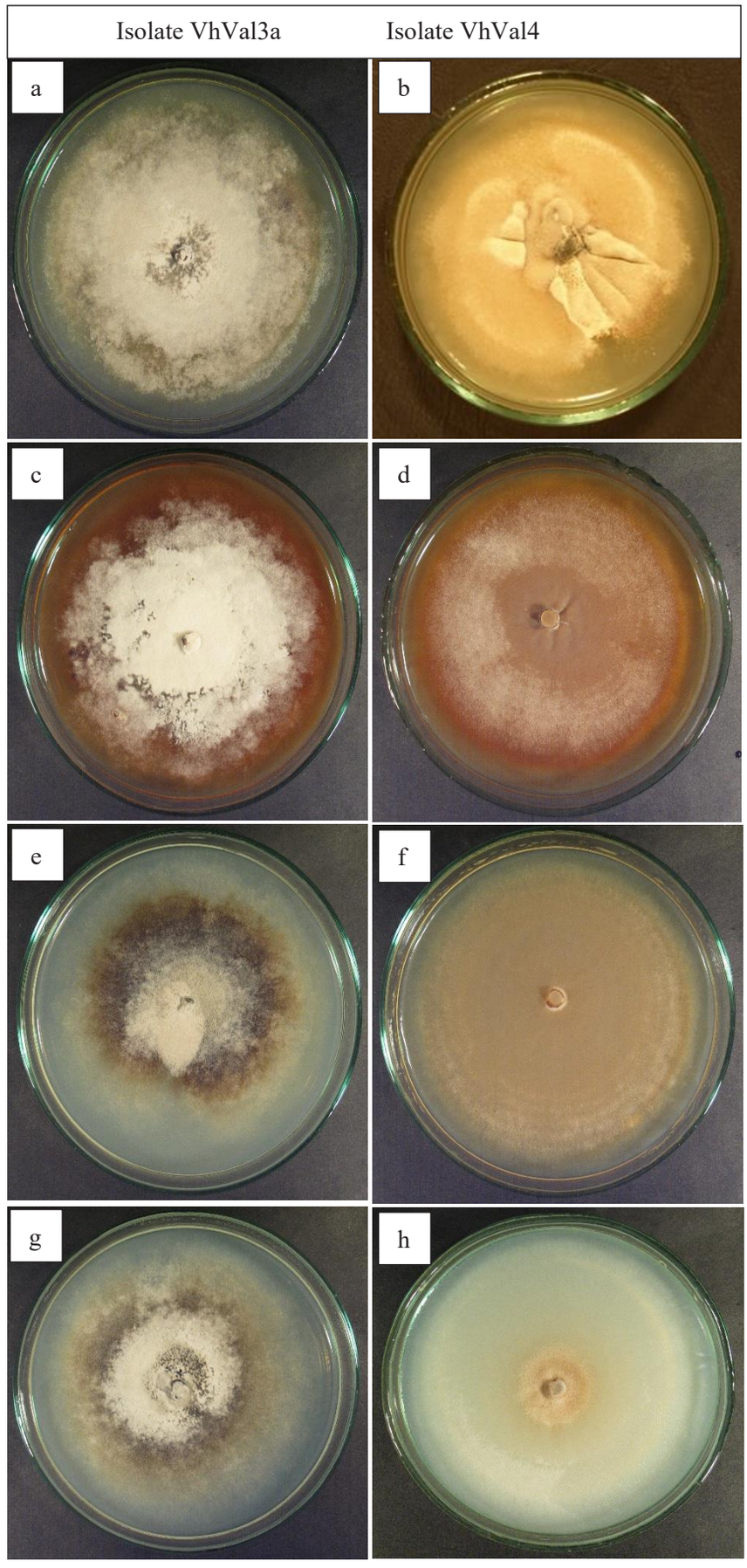

Fig. 4. Variation in the morphology of the fungal cultures of the VhVal3A and VhVal4 *V. heterodoxa* isolates on different media: PDA (a, b), Bor-PDA (c, d), Jag-PDA (e, f), Kon-PDA (g, h)

The cultures of isolates VhVal3A and VhVal4 growing on the WOA medium produced delicate white-cream aerial mycelium on the surface of the medium. At 6 weeks after the inoculation, initially black and fleshy sclerotia, becoming compact and hard with time, were observed on the surface of the medium. The appearance of the colonies growing on the other media modified with WOA (Bor-WOA, Jag-WOA, Kon-WOA) was similar to that of isolate VhVal3A cultured on the WOA medium.

The statistical analysis of the growth dynamics of isolates VhVal3A and VhVal4 showed significant differences in the culture growth on the eight media in each of the incubation conditions (Tab. 3).

Production of *V. heterodoxa* conidia in *in vitro* conditions. The production of *V. heterodoxa* spores was only observed in the diffuse daylight conditions at approx. 20 °C on the OA substrate (variant III). Conidia appeared after 8 days of the experiment. They were visible on the medium surface as initially deli-

Table 3. Comparison of the growth dynamics (mm^2/day) of the *V. heterodoxa* cultures growing on selected media under different lighting conditions (regression line slope coefficient b)

Conditions	Medium							
	Bor-WOA	WOA	Jag-WOA	Kon-WOA	PDA	Bor-PDA	Kon-PDA	Jag-PDA
				Isolate VhVal3A				
Darkness, 19 °C	5.2aA*	5.0aA	6.9abA	7.7abA	8.4abA	9.0bcA	12.1cdA	13.2dA
Photoperiod 12h, 19/12 °C	315.5abB	296.2aB	304.0abB	285.6aB	330.7abB	413.6cB	403.3cC	366.3bcB
Day, 20 °C	306.4aB	307.7aB	311.4abB	310.5abC	363.9bcB	437.9dB	328.3abcB	377.1cB
				Isolate VhVal4				
Darkness, 19 °C	8.4aA	5.3aA	7.2aA	5.6aA	51.7bA	55.2bA	51.8bA	53.6bA
Photoperiod 12h, 19/12 °C	288.5aB	289.9aB	293.6aB	293aB	345.2bcB	420.0dB	310.1abB	358.0cB
Day, 20 °C	313.5aB	299.2aB	310.4abB	282.3abB	360.5bcdB	415.1dB	338.1abcB	380.6cdB

*Homogeneous group according to the Tukey test, p = 0.05
Values in the rows marked with the same lowercase letter do not differ significantly
Values in the columns marked with the same uppercase letter do not differ significantly

Table 4. The number of spores released from the leaf surface of highbush blueberry and bilberry depending on the height of the trapping plate

Distance from the leaf surface (cm)	Number of spores after 4 (pcs)	Number of spores after 9 days (pcs)
1	4	12
5	4	14
10	3	8
15	3	8
20	1	1
25	0	0

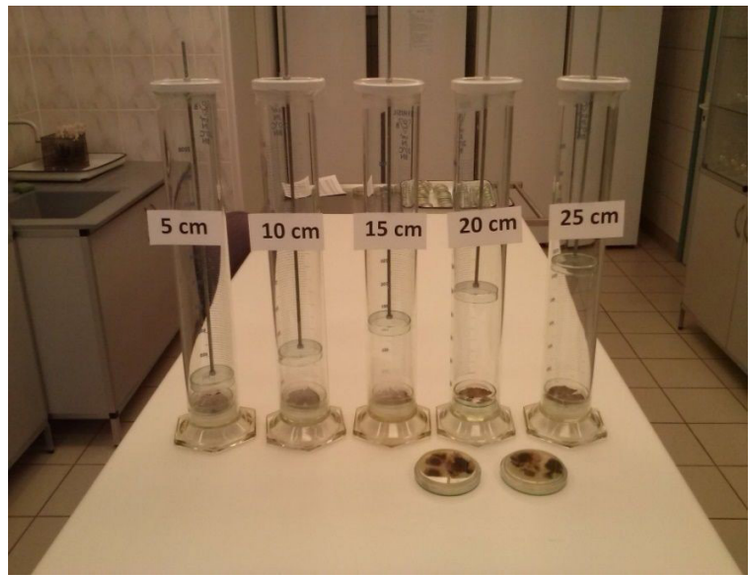

Fig. 5. The release of conidial spores of the fungus *V. heterodoxa* at different heights directly from the leaves of highbush blueberry and bilberry

cate, whitish, aerial mycelium limited by a brown ring composed of interlaced mycelial hyphae.

The conidia produced on the mycelium growing on the OA substrate had a typical appearance for the species *V. heterodoxa*. They were large, 4.5-armed, star-shaped, 200.32–327.77 µm long, and 94.68–158.52 µm wide. The mean dimensions measured in 100 spores were 274.26 (±26.3) µm × 127.29 (±13.87) µm. On day 10 of the experiment, there were 120 spores per plate, and they had a diameter of 100 mm. Isolates VhKir1 and VhK26/2a (bilberry) were found to produce conidia. In turn, isolates VhVal3A, VhVal4, and VhBg/1z/16 (highbush blueberry), isolate VhDM1 (bilberry), and the reference isolates (HBI0401 and DAOMC) did not produce spores in any of the variants tested in this study. In total, 65 out of the 115 *V. heterodoxa* isolates produced conidia in optimal conditions for fungal spore formation.

Release of *V. heterodoxa* conidia at different heights

In the cylinders, single conidia (from 1 to 4) were found on the plates placed at the height of 1, 5, 10, and 15 cm after only 4 days of the experiment. After another 5 days, the number of conidia increased to 14. Their precise number is shown in Table 4.

In the cylinder in which the plate was suspended at a height of 20 cm, a single conidium was found on the cylinder wall at the height of approx. 18 cm (Fig. 5). In turn, no spores were observed in the cylinder with the plate suspended at a height of 25 cm.

DISCUSSION

The fungus *Valdensinia heterodoxa* occurs commonly in Polish forests and is the cause of valdensia leaf blight, a disease of bilberry plants [Siemaszko 1929, Siemaszko 1934, Mułenko and Woodward 1996, Kukuła et al. 2017]. This may be associated with the specific habitat requirements of this pathogen and with the high content of organic matter, which is a source of nitrogen for plants. In bilberry plants growing in the boreal forests in Sweden, the high susceptibility to this disease was correlated with increased concentrations of amino acids, especially glutamine, in host plant leaves [Nordin et al. 1998, Strengbom et al. 2002]. In the present study, a high prevalence of the pathogen was noted every year, mainly in the forests growing in coastal and submontane areas. The conditions in these regions, primarily the high relative air humidity and the large amount of light reaching plants growing in

the lowest parts of the forests, were most probably favourable for the growth of the fungus. *V. heterodoxa* was found to infect bilberry shrubs in Pomorskie (Kopalino, Karwia, Ostrowo) and Małopolskie Voivodeship (Zakopane, Witów, Kiry). The possibility of occurrence of the pathogen in highly humid conditions was also evidenced by its presence in the village of Okuninka (Lubelskie Voivodeship), where infected plants were found in a forest near the lake. High air humidity is one of the main determinants of spore formation and fungal growth [Aamlid 2000, Vogelgsang and Shamoun 2002, 2004, Wilkin 2004, Wilkin et al. 2005a, Wilkin et al. 2005b, Zhao and Shamoun 2006, Annis and Yarborough 2009, Zhao and Shamoun 2010, Abbasi et al. 2023].

To date, the national literature has provided little information on the biology and harmful effects of *V. heterodoxa* and the epidemiology of valdensia leaf blight, probably because the pathogen was found to infect economically unimportant host plant species, e.g. bilberry, lingonberry, or lowbush blueberry. A cue to undertake investigations of the biology and detrimental effects of *V. heterodoxa* and the epidemiology of the disease was the detection of the fungus in the highbush blueberry cv. Jersey in Japan [Nekoduka et al. 2012] and in the highbush blueberry cv. Bluecrop cultivated in a nursery [Dzięcioł et al. 2014] or in the Bluegold cultivar growing in a commercial plantation in Poland [Kukuła et al. 2017].

On the one hand, the present study is a continuation of previous investigations, and on the other hand, the results enlarge the knowledge provided by Kukuła et al. [2017] on the biology of *V. heterodoxa* and the epidemiology of the disease. The research conducted in 18 commercial plantations: eight plantations in Mazowieckie Voivodeship (Prażmów, Piskórka, Wola Żyrowska, Żyrów, Błędów, Huta Błędowska), five in Łódzkie Voivodeship (Paprotnia, Jajkowice, Kłopoczyn), three in Podlaskie Voivodeship (Białousy – two plantations, Sokółka), and two in Lubelskie Voivodeship (Matcze, Horodło) confirmed the presence of *V. heterodoxa* in one of the commercial cultivation of highbush blueberry plants (Prażmów) with high humidity and without chemical control. The commercial plantations selected for the study differed in the location, soil conditions, microclimate, and the age of cultivated plants. The central part of the country is flat or, less frequently, has a southern slope. The soil structure in these plantations was dominated by permeable sandy-peat soils with pH 4–4.5. The soils represented quality class V and/or VI. The planting material consisted of plants purchased in licensed Polish nurseries of fruit trees and shrubs. Plantations with shrubs aged 5–10 years dominated. They were located close to forests or other woodland areas, and one or two of their sides were most often directly adjacent to the forests or were located in forest centers. This suggests that infected bilberry plants may have been a potential source of *V. heterodoxa* infecting the highbush blueberry plants. This hypothesis was verified, as the plants in these plantations had characteristic concentric necrotic spots on their leaves similar to those observed in Japan in a commercial plantation of blueberry cv. Jersey described by Nekoduka et al. [2012] and on blueberry cv. Bluecrop in a planting material nursery reported by Dzięcioł et al. [2014].

In the areas surrounding the commercial highbush blueberry plantations, the occurrence of *V. heterodoxa* was also recorded on lingonberry leaves and non-woody bilberry shoots. Similar findings were reported by Hildebrand and Renderos [2007, 2010, 2012], Hildebrand et al. [2011, 2016], Nekoduka et al. [2012], Lyon [2015], and Abbasi et al. [2023], who found that the fungus infected all green parts of plants, including shoots and unripe berries. In turn, our observations of other plants regarded by other researchers as host plants of the pathogen [Siemaszko 1929, Siemaszko 1934, Bavendamm 1944, Melnik 1981, Norvell and Redhead 1994, Mułenko and Woodward 1996, Melnik 2004, Melnik et al. 2007], i.e. bigleaf maple, downy birch, common hazel, common beech, pedunculate oak, sessile oak, common rowan, wild raspberries, and blackberries as well as such perennials as lily of the valley, wood sorrel, or polygonatum, did not confirm the presence of this fungus on any of these species.

In natural conditions, *V. heterodoxa* conidia are large, macroscopically visible, and resemble 3–4-pointed or sometimes even 5-pointed stars with a size of 400–600 × 100–150 µm [Peyronel 1923, Bavendamm 1944, Peyronel 1953, Redhead and Perrin 1972a, Redhead and Perrin 1972b, Mułenko and Woodward 1996]. In this study, the conidia isolated from the leaves of infected plants (lingonberry, bilberry and highbush blueberry cv. Bluegold) were smaller,

in some cases even by 50%, than those described in the literature. Moreover, the size of the conidia was specific to the host plant species, and the statistical analysis confirmed the significance of these size differences. Fungi from the phylum *Ascomycota* are spread by ascospores and conidia [Kirk et al. 2008]. In many cases, the processes of generation of ascospores and conidia are separated in time. Nevertheless, in some cases, only the imperfect stage occurs commonly in nature, with conidia as the main source of the fungus spread [Marcinkowska 2012], which is the case of *V. heterodoxa*. In the present study, regardless of the host plant, conidia of this fungus on short conidiophores were visible within the spots on the infected leaves, as in the studies conducted by Peyronel [1923], Redhead and Perrin [1972b], Nekoduka et al. [2012], Zhao and Shamoun [2010], and Abbasi et al. [2023]. The unique way of releasing these spores, i.e. through pressure exerted by folding arms on the leaf surface, is considered highly interesting. As suggested by Redhead and Perrin [1972b], this process contributes to the upward ejection of spores to a height of approx. 20 cm. This mechanism has never been recorded, but the ability of conidia to "detach" from the leaf surface was used in this study to acquire single-spore isolates. It was also observed that the placement of the leaves on the Petri dish, with the upper side of the leaf blade facing up, exerted a considerable impact on the frequency and number of produced conidia. They were generated in the characteristic spots formed during the pathogen infection on the upper side of the leaves. During their release, their heads were attached to the medium. The results of the present study indicate that conidia can be released by upward ejection to a height of approximately 18 cm. The active release of spores seems to be a highly effective way of pathogen transmission, which can explain the rapid spread of the disease onto plants.

The development of the disease in natural conditions depends on the weather prevailing in the growing season and the plant vegetation stage. This is closely related to the growth and development of the fungus *V. heterodoxa*. In Canada, the first conidia appeared after overwintering on leaves infected in the previous year; they were formed within sclerotia at high humidity persisting for three consecutive days [Hildebrand and Renderos 2010]. Conidia transferred onto young 3-week-old leaves infected and established parasitic contact with the plant already after 6 hours. The first weak symptoms were observed already in late May or early June, especially when the leaves remained wet for 6–10 hours [Hildebrand and Renderos 2012]. In addition to high humidity, an optimal temperature is required for the growth and development of the pathogen and for colonization of new plants. Conidia infect plant leaves especially quickly in the temperature range of 15–25 °C [Hildebrand and Renderos 2010, Abbasi et al. 2023]. During our observations, the first spots were found on the leaves after abundant rainfall recorded at the beginning of June (at the beginning of the season), and they developed gradually in July.

In their studies, Redhead and Perrin [1972a] found that mycelium growth and pathogen spore formation on artificial media were mainly influenced by the type of media, temperature, and lighting conditions in which Petri dishes with the inoculated pathogen were incubated. Fungal colonies produced spores most efficiently on CM (Cornmeal Agar, Difco) and WOA media incubated at 15 °C for 4–5 days. Vogelgsang and Shamoun [2002] confirmed the strict dependence of fungal spore formation on the temperature and photoperiod. At temperatures <10 °C and without additional lighting (0-hour photoperiod), the growth and development of pathogen colonies were inhibited. In turn, Magnussen et al. [2004] observed the most abundant spore formation at a 12 h day/12 h night photoperiod and a temperature of 16–19 °C. Zhao and Shamoun [2010] reported the most efficient growth and spore formation in a fungus colony on SPDA (Salal-PDA), SOA (Salal-Oatmeal Agar), and WOA media at a temperature of 19 °C (day) and 12 °C (night) and a 12 h day/12 h night photoperiod. The present results indicate that 15–20 °C is the optimum temperature for mycelium growth in laboratory conditions, which is consistent with the findings reported by Hildebrand and Renderos [2012]. Our subsequent analyses showed inhibition of the growth of *V. heterodoxa* colonies on media incubated without access to light, which was also observed by Zhao and Shamoun [2006]. A positive effect was observed when decoctions from highbush blueberry, bilberry, and lily of the valley leaves were added to the media. The analysis of the dynamics of growth of the pathogen colonies on various media and in various light and temperature conditions indi-

cated that, in most cases, both *V. heterodoxa* isolates grew most efficiently on the PDA medium supplemented with leaves from different plants (temp. 20 °C, daylight, and temperature of 19 °C during the day and 12 °C at night with 12-h illumination). The correlation coefficients (r) determined for these isolates were high (above 0.9), which indicates a very strong positive correlation between the size of the fungal colony and the measurement time point in the different incubation conditions.

In the *in vitro* conditions, the colonies of the Polish *V. heterodoxa* isolates exhibited the highest spore production rate on the OA medium with incubation at daylight at 20 °C. The conidia formed in these conditions were smaller than those isolated from the surface of infected leaves collected in natural conditions and conidia obtained by Zhao and Shamoun [2010] on solid media from wheat and rice grains.

Currently, the identification of fungi should be based not only on classical methods but also on molecular techniques, e.g. analyses of species-specific gene sequences or regions, such as the non-coding regions of nuclear DNA – ITS (*Internal Transcribed Spacer*) fragments, which retain polymorphic traits [White et al.1990, Larena et al.1999]. Phylogenetic analyses based on the large subunit rDNA sequence have shown that *V. heterodoxa* forms a separate monophyletic group within the family *Sclerotiniaceae* [Holst-Jensen et al. 1997]. In the present study, the fungal isolates were identified using primers of the ITS region, and a 559-nucleotide product was obtained in the PCR reaction. The BLASTn algorithm-based comparison of the sequence obtained in this study to those available in the GenBank showed its 100% similarity to the sequences deposited under numbers KU306729.1, KF212190.1, AB663682.1, Z81447.1, and KT121733.1, which allowed unambiguous assignment of the studied isolates to the species *Valdensinia heterodoxa*. Based on the nucleotide sequence, a group of 14 isolates from the bilberry shrubs (northern Poland) with a deletion of nucleotide 12 was distinguished.

Valdensia leaf blight caused by the fungus *V. heterodoxa*, initially observed only in New Brunswick province (Canada), is now recorded in neighboring provinces and some US states. Its harmful effects on lowbush blueberry plantations are reflected in a 20 to 60% decrease in yields due to premature defoliation [Hildebrand and Renderos 2010] and disruption of processes responsible for the formation of flower buds for the subsequent season [Ali et al. 2021]. One of the most effective measures to protect plants against this disease in Canada is prevention of pathogen transmission on shoes, clothing, or agricultural machinery wheels [Cornel and Percival 2023]. In Canada, *V. heterodoxa* develops at the same time as other pathogens, e.g. *Botrytis cinerea* [Hildebrand et al. 2001], *Sphaerulina vaccinii* S. Ali, P.D. Hildebrand & P.A. Abbasi, and *Botryosphaeria corticis* (Demaree & Wilcox) Arx & E. Müll. [Ali et al. 2021]. Therefore, to limit its growth, agents used to combat other diseases that pose a high threat to highbush blueberries in Poland, such as anthracnose or gray mold, may prove effective [Bryk et al. 2020].

The presented investigation of the occurrence of *V. heterodoxa* in highbush blueberry plantations in Poland, the study of the dynamics of growth of Polish isolates of the pathogen, the determination of the conditions of conidia production and the height at which staurospores are released, and the molecular characterization of the pathogen isolates are innovative on a national scale and provide more detailed insight into the biology of *V. heterodoxa* and the epidemiology of valdensia leaf blight. The present findings help to be prepared in case of an epidemic of the disease through development and improvement of methods for protection of highbush blueberry plants against the pathogen. Assessing the threat from various diseases and minimizing ecological damage when selecting protection methods is enabled only by thorough knowledge of pathogen biology.

CONCLUSIONS

The following conclusions can be formulated in the present study:

1. Plants belonging to the family *Ericaceae*, including highbush blueberry, are particularly susceptible to *V. heterodoxa* infection.

2. The *V. heterodoxa* isolates originating from the highbush blueberry plants were found to be less capable of spore formation than the isolates obtained from the bilberry plants.

3. In Poland, *V. heterodoxa* is a common pathogen on plants of the genus *Vaccinium*.

4. The fungus exhibits characteristics of a mesophilic and eurythermic species.

5. In axenic cultures the vegetative growth and conidiation of *V. heterodoxa* are strongly influenced by light conditions and the carbon sources provided in the growth medium.

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COMPARISON OF THE COURSE OF PATHOGENESIS CAUSED BY *Verticillium dahliae* (Kleb.) AND *Phytophthora cactorum* (Lebert and Cohn) J. SCHRÖT IN THREE STRAWBERRY CULTIVARS UNDER *in vitro* SELECTION

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ABSTRACT

The strawberries are more or less susceptible to *Verticillium dahliae* and *Phytophthora cactorum*, that's why a constant need to expand knowledge about the mechanisms of resistance to diseases caused by these fungi is requied. In the present study, the course of pathogenesis caused by *V. dahliae* and *P. cactorum* in three strawberry cultivars, *i.e.*, Elsanta, Feltar and Teresa, was compared under *in vitro* selection. The results showed that the extent and rate of development of disease symptoms were mostly insignificantly differentiated on subsequent observation dates. The resemblance observed in Verticillium wilt and phytophthorosis course within each microclone suggested the occurrence of a similar genetic mechanism of resistance response to both fungi. The likeness of average susceptibility to both pathogenic fungi evaluated with McKinney Disease Index (DI%) corresponded with the genetic similarity on the DNA level, which was estimated between selected submicroclones resistant to pathogens within each microclone. The microclone most susceptible to *V. dahliae* and to *P. cactorum* was Teresa (DI = 34.02%; 43.53%, respectively), whereas the lowest susceptibility to *V. dahliae* was observed in Elsanta microclone (DI = 29.85%). The microclone Feltar was considered to be the least susceptible to *P. cactorum* (DI = 19.50%). Moreover, a strong positive correlation was observed for the extent as well as for the rate between the development of both diseases in each microclone. Heritability in a broad–sense (h^2_{bs}) of the extent and rate of development of both pathogeneses reached values above 70%, which revealed the strong genetic determination of the resistance response to both pathogens in the analysed cultivars.

Keywords: *Fragaria* × *ananassa*, Verticillium wilt, phytophthorosis, resistance, DNA analysis, heritability, correlation

INTRODUCTION

The strawberry (*Fragaria* × *ananassa* Duch.) remains one of the most important soft fruits cultivated worldwide. Due to its many essential nutrients, this fruit is a staple in the diets of millions, and it is cultivated in various regions of the world, from the Arctic to the tropics [Hummer and Hancock 2009, Mukherjee and Gantait 2024].

In 2022, the global strawberry production amounted to 9.5 million tonnes from approximately 397,000 ha. The largest fruit producers are China (3.35 million tonnes) and the United States (1.26 million tonnes), followed by Turkey (728 thousand tonnes), Egypt (637 thousand tonnes), Mexico (568 thousand tonnes), Spain (325 thousand tonnes) and Russia (254 thou-

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sand tonnes). Poland's strawberry production in 2022 amounted to 199,000 tonnes, on an area of 31,000 ha [FAOSTAT 2024].

The susceptibility of strawberry plants to common biotic stress factors causes significant yield reductions and considerable economic losses [Wijerathna-Yapa and Hiti-Bandaralage 2023]. The main phytopathogens found on European strawberry plantations are the following fungi: *Phytophthora cactorum*, which causes leather crown rot and leather fruit rot in strawberries; *Verticillium dahliae*, which causes Verticillium wilt; *Colletotrichum acutatum*, which causes anthracnose; *Sphaerotheca macularis*, which causes powdery mildew in strawberries; *Phytophthora fragariae* var. *fragariae*, which is responsible for red root rot in strawberries; *Botrytis cinerea*, which causes grey mould on fruits; and *Alternaria alternata f. sp. fragariae*, which causes leaf spot [Parikka 2004, Olbricht and Hanke 2008, Hu et al. 2023, Jiménez et al. 2023, Alam et al. 2024a].

Some of the most dangerous diseases found on strawberry plantations include crown rot of strawberry rhizome and leather rot of strawberry fruit [Hantula et al. 2000]. Both of these diseases are caused by the fungus of the genus *Phytophthora*, specifically by its specialised form of *Phytophthora cactorum*, which is a polyphagous organism commonly found in soils of the temperate climate zone. It causes a disease called phytophthorosis in 200 species of cultivated plants from 60 families [Ribeiro 1978, Hantula et al. 2000, Bielenin 2002, Horst 2008, Orlikowski et al. 2012, Garrido et al. 2016, Orlikowski et al. 2017]. Ellis et al. [1998] report that the average losses due to diseases on strawberry plantations in the USA amount to 20–30%, and during a particularly severe outbreak of the disease in 1981 in the state of Ohio, the losses were as high as 50%. As reported by Bielenin [1999], losses in strawberry seedlings due to crown rot, on a disease-affected plantation, reached 40% in 1994, whereas in the years 1995–1997, due to leather fruit rot on the Elsanta, Senga Sengana and Syriusz cultivars, losses in fruit reached from 20 to 80%.

Another dangerous soil-borne disease that affects strawberries is Verticillium wilt, which is caused by the fungus *Verticillium dahliae* (Kleb.) [Kurze et al. 2001, Masny and Żurawicz 2008, Żebrowska 2010, Sowik et al. 2016]. The fungus *Verticillium dahliae* is a polyphagous fungus widespread throughout the world, which is particularly dangerous in temperate and tropical climates [Sanei et al. 2008]. It causes diseases in more than 300 species of cultivated and ornamental plants [Profic-Alwasiak 2000, Meszka et al. 2005, Fradin and Thomma 2006, Żebrowska et al. 2006, Meszka and Bielenin 2009, Nouri et al. 2012, Masny et al. 2014, Özer and Bayraktar 2016]. Meszka and Bielenin [2009] report that losses on strawberry plantations, caused by the occurrence of *V. dahliae* under conditions favourable to its development, can account for up to 80%. Shaw et al. [2005, 2010] state that plant mortality caused by the presence of a pathogen in the soil on plantations of particularly susceptible strawberry cultivars reaches up to 75%.

One of the key resistance breeding goals for this species is to develop strawberry cultivars with increased resistance to naturally occurring pathogens. Developing cultivars that are less susceptible or resistant to pathogens contributes to a significant reduction in the use of plant protection products in cultivation, which is harmful to humans and the environment, and thus to a reduction in production costs [Zurn et al. 2020]. The fungicides currently approved for use are not highly effective, or their use is not economical. In some countries, they are being withdrawn from the market, and their continued use is prohibited due to their harmful effects on the environment [Antanaviciute et al. 2015]. One should also expect an increase in the pathogens' resistance to frequently used chemicals.

The breeding work to improve the genetic resistance of the strawberry is greatly facilitated by the use of modern biotechnological methods, *i.e. in vitro* selection, or a comparison of cultivars at the molecular level [Mukherjee and Gantait 2024]. One of the most useful methods is the selection process in tissue cultures. In a compact laboratory space, numerous plants can be selected based on biotic and abiotic factors. Selection conducted under *in vitro* conditions, performed under controlled environment, is highly effective [Rai et al. 2011, Wijerathna-Yapa and Hiti-Bandaralage 2023]. The plants with increased resistance to the selective factor, obtained in this way, can be used for further breeding research as a source of resistance genes.

The strawberry's mechanisms of resistance to *V. dahliae* and *P. cactorum* are not fully understood

or explained. Little is known about the genetic factors that mediate resistance to these pathogens [Zurn et al. 2020]. Lynn et al. [2024] report that the strawberry's resistance to powdery mildew is polygenically controlled. To date, several genes associated with the strawberry's resistance to various pathogens have been discovered. Ma et al. [2023] write that the *FaMBL1* gene plays an important role in the strawberry's response to fungal diseases caused by *Colletotrichum fioriniae* and *Botrytis cinerea*. The loci *FaRMp1* and *FaRMp2*, *FaRMp3* are associated with partial resistance to *Macrophomina phaseolina* [Alam et al. 2024b]. Amil-Ruiz et al. [2011] report that, in response to biotic stress, strawberries exhibit responses similar to those of other plants. It is assumed that this plant recognises pathogens and responds accordingly, based on innate immunity, which comprises all cellular and molecular mechanisms that the plant has to defend itself against pathogens. According to recent research into the *Fragaria* × *ananassa* genome, 247 *WRKY* transcription factors have been identified. Genes from the *WRKY*TF family are known for their various roles in the resistance response of several plant species to biotic and abiotic stress [Garrido-Gala et al. 2022, Vondracek et al. 2024]. There is a great need for further research to identify the mechanisms of resistance to pathogens infecting strawberries to support further breeding work.

The present paper compared the susceptibility of three strawberry cultivars to infection by two pathogenic fungi, *i.e. Verticillium dahliae* and *Phytophthora cactorum*, and the course of pathogenesis under *in vitro* culture conditions. The study determined correlations between susceptibility to Verticillium wilt and phytophthorosis, and estimated the genetic determination of susceptibility of the cultivars tested to pathogens using the heritability coefficient.

MATERIALS AND METHODS

Preparation of starting material for *in vitro* selection

The strawberry cultivars used in the experiment included Elsanta (Gorella × Holiday), Feltar ((Senga Tigaiga × Merton Dawn) S_1) and Teresa (Redgauntlet S1 × Senga Sengana S1), originated from the collection of the Department of Genetic and Horticultural Plant Breeding at the University of Life Sciences in Lublin. The Dutch variety Elsanta is widely known to be susceptible to root diseases caused by soil-borne pathogens. The range, rate, and course of disease development in other varieties have not yet been investigated.

From these donor plants, 3–4-cm-long terminal sections of young stolons, along with a node containing meristematic tissue, were collected (approximately 50–60 units from each cultivar) to propagate the cultivars in an *in vitro* culture. The stolons were rinsed for 30 minutes under running water, and then in a disinfectant solution (200 mL sterile distilled water, 2 mL sodium hypochlorite, 2–3 drops of wetting agent, *i.e.* a liquid detergent) for 15 minutes.

For the *in vitro* regeneration of explants, Murashige and Skoog medium (MS medium) was used [Murashige and Skoog 1962]. The medium was supplemented with 1 mg × dm^{-3} IAA (indolyl-3-acetic acid), 1 mg × dm^{-3} BAP (6-benzylaminopurine), and 0.01 mg × dm^{-3} GA_3 (gibberellic acid, tertiary derivative), and solidified with 7.5 g × dm^{-3} agar, pH 5.7. The medium was decontaminated in an autoclave at 121 °C, under a pressure of 0.1 MPa, for 20 min.

The surface-decontaminated explants were individually placed into test tubes with MS medium enriched with growth regulators, under aseptic conditions, under a laminar horizontal air flow chamber (type: KL-21, Polon, Poznań, Poland). The culture was maintained for 5–6 weeks under controlled environmental conditions (phytotron – temperature of 20 °C, a photoperiod of 16 hours' day / 8 hours' night, light intensity of 30 µmol × m^{-2} × s^{-1}).

After that time, the obtained microshoots were transferred, under aseptic conditions, onto the proliferating MS medium by adding growth regulators (step II of proliferation). The proliferating cultures were divided and transferred onto a fresh MS medium without growth regulators so that the obtained microshoots could take root. The culture was maintained until the microshoots were well rooted and reached at least the 4-leaf stage. In this way, three microclones Elsanta, Feltar, and Teresa were obtained. Each microclone consisted of 300 microplants which served as starting material for *in vitro* selection.

Preparation of the selective factor

The experiment used two phytopathogenic soil-borne fungi, *i.e. Phytophthora cactorum* (Lebert and Cohn)

J. Schröt and *Verticillium dahliae* Klebahn. Pure phytopathogen cultures (*Phytophthora cactorum*, catalogue No 1559 and *Verticillium dahliae* catalogue No 1093) were acquired from the Bank of Pathogens in Poznań (Poland). The pathogens were cultured on the Potato Dextrose Agar (PDA) medium containing 300 g × dm^{-3} potatoes, 20 g × dm^{-3} agar and 20 g × dm^{-3} glucose in a distilled water solution. The medium was decontaminated in a pressure autoclave at 121 °C, under a pressure of 0.1 MPa for 20 minutes. Then, 100 mg × dm^{-3} streptomycin was added to the medium under sterile conditions, after which it was poured into aseptic Petri dishes and left to solidify. The pathogen was inoculated onto PDA medium under a laminar air flow chamber and cultured in the dark for 3 weeks at 18–20 °C. Three-week-old pathogen cultures were used to prepare a homogenate of live mycelium, serving as an inoculum for infecting the microplants. Under sterile conditions, the pathogen cultures were flooded with 50 ml of sterile distilled water. The suspension was then homogenised and diluted with sterile distilled water at a ratio of 1 : 10 V/V to obtain an appropriate spore concentration (10^5 × mL^{-1}).

In vitro culture selection

Well-rooted microplants of each microclone, at least at the stage of four leaves, were the starting material for the selection. The plants were inoculated, under aseptic conditions, by immersing them in the inoculum for 1 minute, after damaging the roots by cutting them with a scalpel to a length of approximately 1.5 cm. The inoculated plants were placed into Petri dishes with a pre-prepared agar medium without minerals and sucrose (7.5 g agar dissolved in 1000 mL distilled water, pH 5.7). The experiment was set up in three repetitions for each microclone and for each pathogen. One hundred plants were used for a single repetition. Microplants within each microclone were divided into two groups. One group was infected by *Verticillium dahliae* creating 'submicroclone V.d.'. The second group was infected by *Phytophthora cactorum* creating 'submicroclone P.c.' A control sample was also prepared for each microclone, where well-rooted plants at a stage of at least 4 leaves, after damaging the roots (by cutting them with a scalpel to a length of approximately 1.5 cm), were immersed in sterile distilled water for 1 minute (mock inoculation). The plants were then placed into the Petri dishes with agar medium. For each microclone, the control comprised 100 plants. The experiment used a total of 3,200 plants.

Observation of the degree of plant infection

According to the methodology provided by Żebrowska et al. [2006] and Żebrowska [2011], the development of disease symptoms was observed on five observation dates: date I – 15 days after inoculation; date II – 30 days after inoculation; date III – 45 days after inoculation; date IV – 60 days after inoculation; date V – 75 days after inoculation.

According to the methodology provided by Sowik et al. [2001], Żebrowska [2011], and Sowik et al. [2015], the degree of plant infection was assessed on a five-point valuation scale, where: 0 – plants without infection symptoms (100% resistance); 1 – infection affecting 1 leaf (25%); 2 – infection affecting 2 leaves (50%); 3 – infection affecting 3 leaves (75%); 4 – infection affecting 4 or more leaves, or totally affected plants (100% susceptibility).

The course of pathogenesis in the microclones tested after being infected *in vitro* by *V. dahliae* and *P. cactorum* was assessed at successive observation dates using disease indices (DI) for the range (DI%) [McKinney 1923] and rate of infection development (DIp) [Simmonds 1987]. Using the mean value of the infection range index (DI%), susceptibility of the microclones to pathogens was determined as well.
The disease index for the infection development range (DI%) was calculated using the following formula:

$$DI(\%) = (\Sigma vn)/(NV) \times 100$$

where:
DI(%) – disease index,
v – numerical value of the infection class,
n – number of plants at a particular observation date in a particular class,
N – total number of infected plants in a particular sample,
V – numerical value of the highest class.
'Class' is a disease rating scale of McKinney formula which is used for Disease Index (DI) evaluation (McKinney 1923). The values of this parameter are given in McKinney publication [1923].

The rate of infection development over time (DIp) was determined using the disease index calculated according to the following formula:

$$DIp = p \times I\,(1 - I)$$

where:
DIp – rate of infection development,
I – proportion of plants (%) with symptoms of infection at a particular observation date in a particular class,
p – pathogen reproduction rate; for susceptible cultivars, it was assumed that $p = 1$.

In addition, within each microclone, the following were assessed for the range and rate of pathogenesis development:

1) correlations using Pearson's linear correlation coefficient (r);

2) heritability in a broad sense (h^2_{bs}) (according to the formula $h^2_{bs} = V_G/V_p$) [Falconer and Mackay 1996], where:
h^2_{bs} – heritability in a broad sense,
V_G – genetic variation (including effects due to dominance and epistasis),
V_p – phenotypic variation.

The heritability coefficient values were estimated based on genetic interpretation of statistical components of variance for the single classification system (one-factor analysis of variance (ANOVA)). The numerical data were statistically analysed using the Statistica 13.1 program [2020]. The significance of the differences between the values of the examined traits was estimated with the Student's t-test and Duncan's multiple range test at $P \leq 0.05$.

DNA isolation, ISSR PCR and electrophoretic separation of the obtained products

Once the observations were completed, within each microclone, DNA was isolated from ten randomly selected plants (submicroclones) resistant to pathogenic selective factors, using the modified CTAB method described by Gawel and Jarret [1991]. DNA purity and concentration were determined using a Thermo Scientific NanoDrop 2000 spectrophotometer.

Inter simple sequence repeat (ISSR) markers were used to examine the genetic similarity, at the DNA level, of forms resistant to the two pathogens. The experiment analysed 16 ISSR markers provided by Sigma-Aldrich.

DNA amplification was carried out in a thermocycler (TProfessional Basic Gradient Biometra GmbH) at a final reaction volume of 15 µL for each reaction, which contained 1.5 µL PCR buffer (Dream Taq Buffer, Thermo Scientific), 1.2 µL dNTP (10 mM dNTP MIX, Thermo Scientific), 0.7 µL oligonucleotide primer, 0.9 µL $MgCl_2$ (25 mM, Thermo Scientific), 0.15 µL Taq DNA polymerase (Dream Taq DNA polymerase 5 U/µL, Thermo Scientific) and 3 µL DNA template. Each of the 35 polymerase chain reaction (PCR) cycles comprised 3 steps: 45 seconds at 94 °C (DNA denaturation); 1 minute at the annealing temperature; 2 minutes at 72 °C (DNA elongation). After 35 cycles, the samples were maintained at 72 °C for 7 minutes to carry out the final elongation step. The annealing temperature was adjusted to the melting temperature (T_M) for the primers used in the reaction. In order to check repeatability, the primers used in the experiment were tested twice on the same sample.

The ISSR PCR reaction products obtained were separated by electrophoresis on 1.5% agarose gel containing 0.1% of ethidium bromide in 1 × TBE buffer. The electrophoresis was carried out for 90 minutes at a voltage of 100 V. DNA fragments stained with ethidium bromide were visualised under ultraviolet light (UV). Photographs of the gel were taken using GeneSnap Syngene. Further analysis of the images was carried out using the GeneTools Syngene program. Based on the results obtained, a dendrogram was generated in the Past5 program, using the UPGMA (Unweighted Pair Group with Arithmetic Mean) method, showing the genetic similarity of the resistant plants at the DNA level.

RESULTS

Course of pathogenesis

By analysing the course of pathogenesis in the microclones after being infected by *P. cactorum* or *V. dahliae*, it was found that the intensity of microplants' dieback varied, and the range and rate of disease development were impacted by the microclone tested. Gradual chlorosis of the leaves occurred on plants inoculated with mycelium homogenate, result-

ing in dieback of entire microplants. The first symptoms of phytophthorosis and Verticillium wilt on the microplants became visible 15 days after inoculation.

As for the Elsanta microclone (Fig. 1), no significant differences were noted between the mean range (DI%) of Verticillium wilt and phytophthorosis at successive observation dates. Significant differences between the mean range of Verticillium wilt and phytophthorosis were found for the Feltar microclone (Fig. 2) only at observation dates II and III. In contrast, in the Teresa microclone (Fig. 3), the differences between the range of pathogeneses were insignificant at observation date I, whereas at the subsequent observation dates (II–V), these differences proved to be significant.

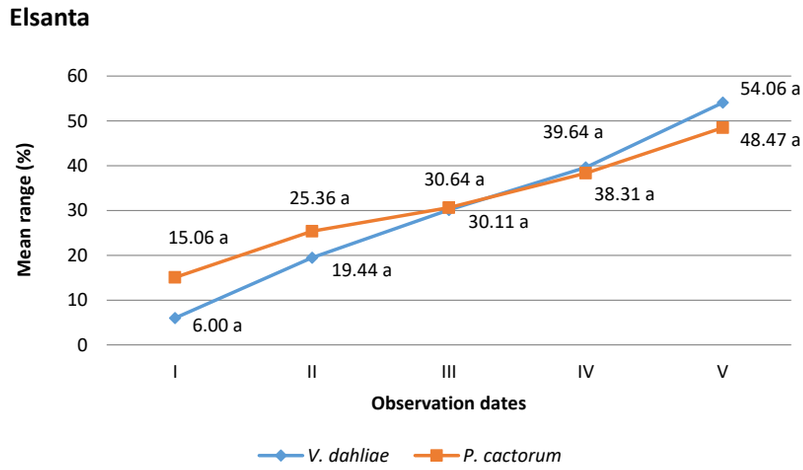

Fig. 1. Differences for the mean range of pathogenesis (%) in the Elsanta microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

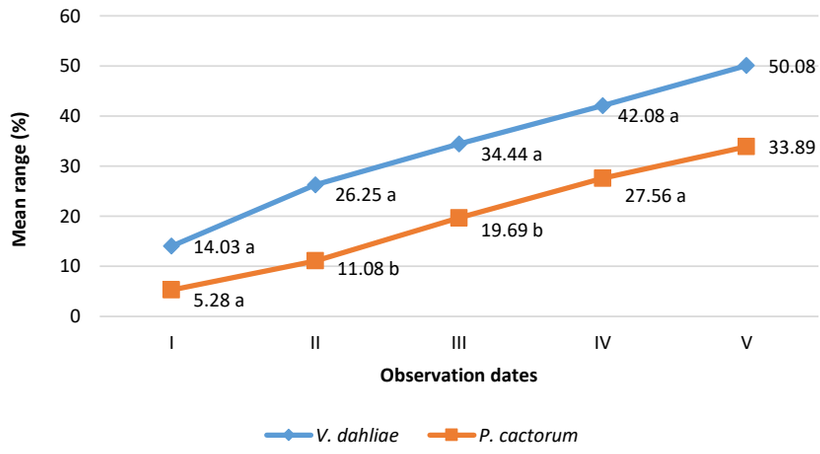

Fig. 2. Differences for the mean range of pathogenesis (%) in the Feltar microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

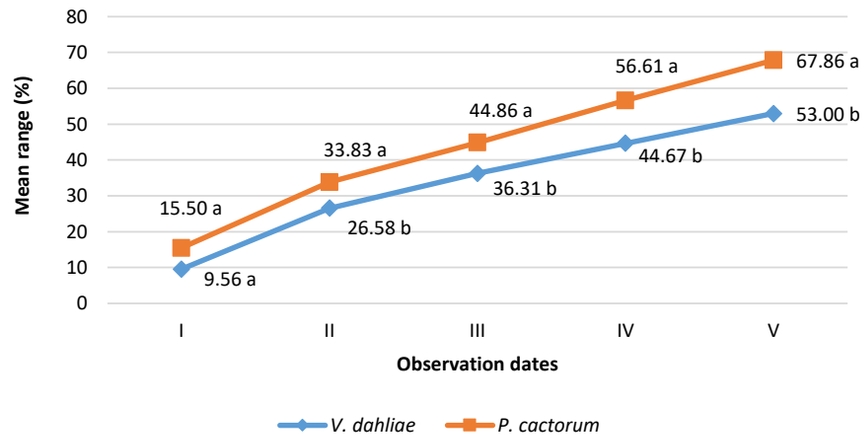

Fig. 3. Differences for the mean range of pathogenesis (%) in the Teresa microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

Table 1. Differences between the mean range (%) of Verticillium wilt and phytophthorosis in the microclones tested. The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

	Elsanta	Feltar	Teresa
V. dahliae	29.85 a	33.38 a	34.02 a
P. cactorum	31.57 a	19.50 b	43.53 a

Statistical analysis (ANOVA) showed the occurrence of significant differences between the total mean range of both diseases only in the Feltar microclone. As for the Elsanta and Teresa microclones, these differences were insignificant (Tab. 1).

By analysing the differences in the rate of development (DIp) of Verticillium wilt and phytophthorosis at successive observation dates, it was concluded that they were only significant for the first observation date in the Elsanta microclone (Fig. 4). In the Feltar (Fig. 5) and Teresa (Fig. 6) microclones, no significant differences were noted in the rate of development of phytophthorosis and Verticillium wilt at successive observation dates.

No significant difference was noted between the total mean rate of development of Verticillium wilt and phytophthorosis in all the microclones tested (Tab. 2).

Correlations

The correlations for the range and rate of development of Verticillium wilt and phytophthorosis, estimated using correlation (r_{xy}) and regression (b_{yx}) coefficients, are provided in Tables 3 and 4 (resp.).

Heritability

The calculated heritability coefficients in a broad sense (h^2_{bs}) for the range of development of Verticillium wilt and phytophthorosis reached values of 85.04% and 97.97%, respectively, while for the rate of development, 75.04% and 77.90%, respectively.

Molecular analyses

Molecular analyses involved determining genetic similarity assessed by cluster analysis (UPGMA), based on DNA polymorphism identified in selected

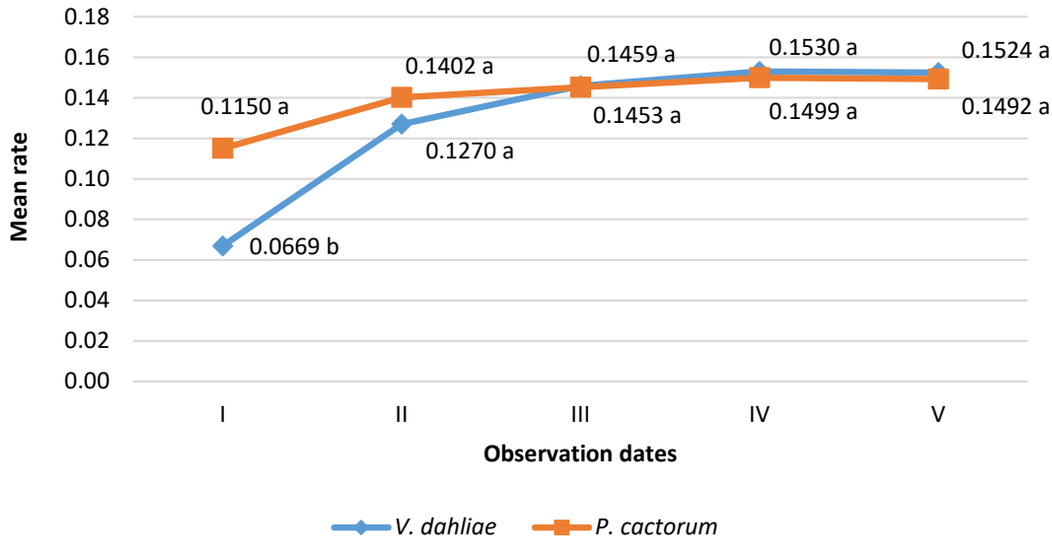

Fig. 4. Differences for the mean rate of infection development in the Elsanta microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

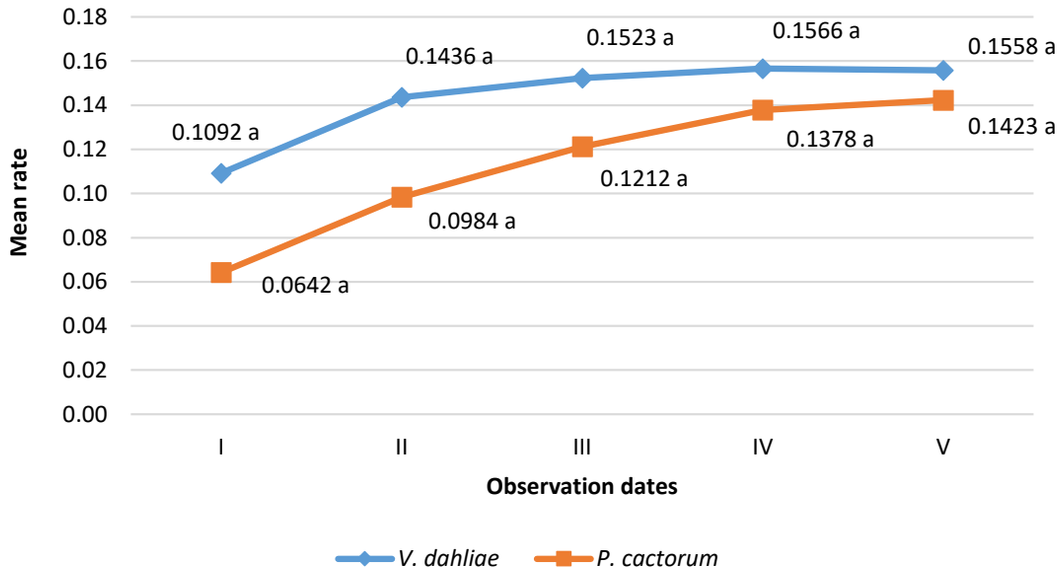

Fig. 5. Differences for the mean rate of infection development in the Feltar microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

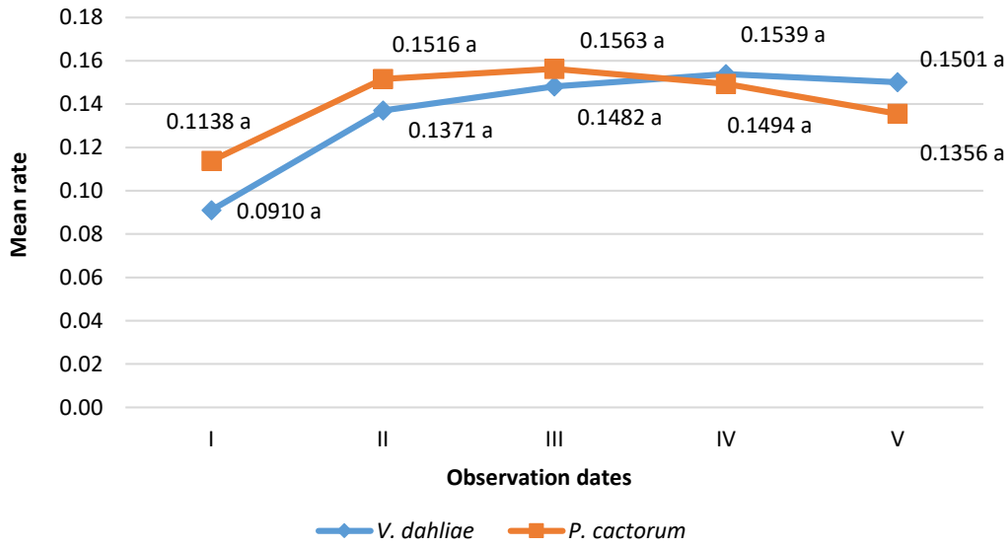

Fig. 6. Differences for the mean rate of infection development in the Teresa microclone after being infected by *P. cactorum* and *V. dahliae* at successive observation dates (I–V). The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

Table 2. Differences between the total mean rate of development of Verticillium wilt and phytophthorosis in the microclones tested. The mean values marked with the same letter do not differ significantly at $P \leq 0.05$

	Elsanta	Feltar	Teresa
V. dahliae	0.1290 a	0.1435 a	0.1360 a
P. cactorum	0.1399 a	0.1128 a	0.1414 a

Table 3. Correlations between the range of development of Verticillium wilt and phytophthorosis in the strawberry microclones tested

	Elsanta	Feltar	Teresa
r_{xy}	0.7503*	0.7748*	0.9876*
R^2	0.5630	0.6000	0.9753
1) b_{yx}	0.6295	0.7353	0.8088
2) b_{yx}	0.8943	0.8164	1.2060

r_{xy} – Pearson's linear correlation coefficient; * – correlation significant at $P \leq 0.05$; R^2 – determination coefficient; b_{yx} – linear regression coefficient, where: 1) x – development of Verticillium wilt, y – development of phytophthorosis; 2) x – development of phytophthorosis, y – development of Verticillium wilt

plant material using ISSR markers. The material consisted of microplants which withstood the pressure of selective factors by forming pathogen-resistant submicroclones within each microclone. For 8 out of the 16 ISSR primers tested, amplification products were obtained (Tab. 5). A single primer was involved in the synthesis of three (primer 11) to eight (primer 8) – an average of 5.9 – polymorphic products (Tab. 5).

For eight primers analysed, 52 DNA fragments were obtained, of which 47 (89.58%) were polymorphic. The size of the sequences obtained for individual ISSR primers ranged from 300 to 3,500 base pairs (Tab. 5). Primer 14 allowed all the cultivars (microclones) analysed to be distinguished from each other.

Analysis of the UPGMA dendrogram, plotted on the basis of the similarity matrix, enabled the determination of genetic similarity between the analysed submicroclones resistant to Verticillium wilt and phytophthorosis (Fig. 7). The presence of two main cluster groups was identified. The first group (I) and the second group (II) were 57% similar to each other.

In the first (I) group, submicroclones Elsanta, resistant to *V. dahliae* and *P. cactorum*, which were characterised by 88% genetic similarity, were most similar to each other. A comparable high similarity was demonstrated between the Teresa submicroclones (84%). The

Table 4. Correlations between the rate of development of Verticillium wilt and phytophthorosis in the strawberry microclones tested

	Elsanta	Feltar	Teresa
r_{xy}	0.9981*	0.9511*	0.8373
R^2	0.9962	0.9046	0.7011
1) b_{yx}	0.3970	1.5408	0.5559
2) b_{yx}	2.5086	0.5871	1.2612

Explanation as in Table 3

Table 5. Assessment of the polymorphism of the strawberry genome tested using ISSR primers

Number of primer	Sequence (5'–3')	Number of loci			
		Total	Polimorphic	%P	Size range (bp)
1	VBVACACACACACACAC	7	7	100.0	400–2000
7	HVHTGTTGTTGTTGTTGT	6	5	83.3	600–3000
8	BDBCACCACCACCACCAC	8	8	100.0	300–1500
10	GAAGAAGAAGAAGAAGAA	5	5	100.0	1000–1500
11	ATGATGATGATGATGATG	6	3	50.0	300–3000
13	GATAGATAGATAGATAGATA	7	7	100.0	700–3500
14	GACAGACAGACAGACAGACA	6	5	83.3	300–2500
16	AGTGAGTGAGTGAGTG	7	7	100.0	300–2500
	Mean	6.5	5.9	–	–
	Totality	52	47	89.58	300–3500

Explanation of symbols: H = A + T + C, B = G + T + C, D = G + A + T, V = G + A + C
%P – percentage of polymorphism

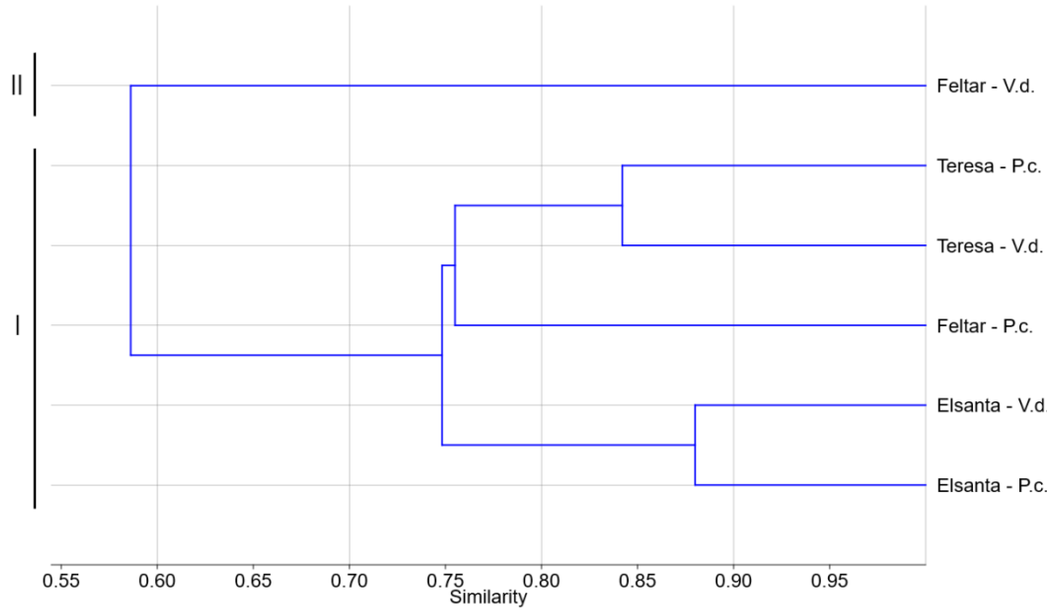

Fig. 7. UPGMA dendrogram generated in the Past5 program, showing the genetic similarity between the analysed strawberry submicroclones resistant to phytophthorosis and Verticillium wilt

Legend for the dendrogram:
Elsanta; Feltar; Teresa – P.c. – submicroclone resistant after being infected by *Phytophthora cactorum*
Elsanta; Feltar; Teresa – V.d. – submicroclone resistant after being infected by *Verticillium dahliae*

Feltar submicroclone, resistant to *P. cactorum*, exhibited 76% similarity to the analysed Teresa submicroclones.

DISCUSSION

Verticillium wilt and phytophthorosis caused by soil-borne pathogenic fungi, *i.e.*, *Verticillium dahliae* and *Phytophthora cactorum*, are very serious, non-chemically controlled infectious diseases of many plant species, including strawberries. An important factor limiting the occurrence of these diseases on strawberry plantations is the cultivation of cultivars with genetically determined resistance to these diseases.

Selection aimed at obtaining new strawberry cultivars, resistant or tolerant to *Verticillium dahliae* or *Phytophthora cactorum*, is currently one of the major breeding directions for this species in Poland and worldwide. Sowik et al. [2008] and Żebrowska [2010], thanks to the application of selection on the pathogenic fungus *Verticillium dahliae* in an *in vitro* culture, examined the resistance of certain strawberry cultivars and breeding clones to this pathogen. Żebrowska [2010] concluded that the susceptibility of the tested strawberry cultivars under *in vitro* conditions was similar to the susceptibility of these cultivars under field conditions, and that the selection in an *in vitro* culture can be successfully used in breeding programmes. It should be emphasised that the defensive response of plants, observed during *in vitro* selection under constant and controlled environmental conditions, reflects the genetically determined host-pathogen interaction. The use of *in vitro* eliminates variable environmental conditions that can affect a plant's genetically determined resistance to stress factors. This makes *in vitro* selection a highly valuable method for studying the genetic mechanisms behind plant resistance. The results obtained under these conditions, regarding the course of pathogenesis, are completely reliable and provide accurate information on the genetic resistance of plants to pathogens.

In the present study, each microclone responded differently to two pathogens, which was due to the genetic differences between the cultivars from which

the microclones were derived. In contrast, the similar course of phytophthorosis and Verticillium wilt observed in each microclone, along with the slight and mostly insignificant differences in the range and rate of development of both diseases at successive observation dates, suggests that there may be similar mechanisms of genetic resistance to these pathogens. Thirty days after infection, there was a noticeable slowdown in the rate of infection development. In addition, the high genetic similarity of over 80% existing between submicroclones with a similar susceptibility to both pathogens (*e.g.* Elsanta and Teresa) also suggests that similar genetic defence mechanisms are operating in these cultivars. However, the significant difference found in the Feltar microclone in the susceptibility to both pathogens was justified by the lower genetic similarity at the DNA level (75%) between the submicroclones resistant to phytophthorosis and Verticillium wilt. The high and positive correlations, estimated for both the range and rate of development of pathogenesis in the analysed microclones, and the high heritability coefficient of these traits further confirm the activation of similar genetic defence mechanisms in plants in response to infection by *V. dahliae* and *P. cactorum*.

The results obtained in the present study suggest that there is no single defined resistance mechanism within the *Fragaria* × *ananassa* species, and that the plant-pathogen response is controlled by numerous factors, including genetic factors. Parikka [2003], when testing strawberry cultivars cultivated in Finland in terms of their susceptibility to phytophthorosis, noted that the susceptibility degree in particular cultivars can vary depending on the season in which the infection occurred. Resistance to *P. cactorum* was high in the plants during winter, but decreased during summer. Eikemo et al. [2003] report that the degree of resistance can also be affected by the plant's age and physiological condition.

The existence of differences in the susceptibility of a cultivar to different pathogens is confirmed by a study by Pérez-Jiménez et al. [2012]. The researchers tested the resistance/susceptibility of several strawberry cultivars cultivated in Spain to *Phytophthora cactorum*, *Verticillium dahliae* and two *Xanthomonas fragariae* strains (IVIA 349.94a and NCPPB 1469). Based on the results obtained, they concluded that none of the tested cultivars was, to a comparable degree, resistant to all selective factors. The Sieger cultivar, tolerant to both *P. cactorum* and *V. dahliae*, was resistant to the two tested *X. fragariae* strains. The Aguedilla cultivar, resistant to the IVIA 349.94a strain of *X. fragariae*, and tolerant to the NCPPB 1469 strain of *X. fragariae* as well as to *P. cactorum*, appeared to be susceptible to *V. dahliae*.

Shokaeva et al. [2011], when assessing the susceptibility of several strawberry somaclones to *Botrytis cinerea*, *Phytophthora cactorum* and salination, found that there were differences in susceptibility to the selective factor among the somaclones tested. Similar conclusions were reached by Eikemo et al. [2003], who examined the susceptibility of 26 strawberry cultivars to phytophthorosis. The results of their study show that resistance to *P. cactorum* varies greatly between cultivars. Zurn et al. [2020], when assessing different strawberry genotypes for their resistance to *Verticillium dahliae*, *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *fragariae*, found different levels of resistance to the pathogens tested within the cultivars and breeding clones. Based on the above results, it can be concluded that within the *Fragaria* × *ananassa* species, there is no single pathogen resistance mechanism, rather it largely depends on the cultivar's origin. Eikemo et al. [2003] and Schafleitner et al. [2013] report that resistance to *P. cactorum* appears to be polygenic, but not all factors affecting it are known. The results obtained in the present study, and the results obtained by other authors, suggest that each cultivar responds differently to a particular selective factor. They also suggest the lack of uniform resistance to many selective factors within the cultivar, *i.e.* polygenicity of resistance.

The correlations found in the present study for the range and rate of development of Verticillium wilt and phytophthorosis, estimated using correlation and regression coefficients, indicated the possibility of mutual prediction of the course of these diseases in the strawberry. The greatest predictable intensity of phytophthora symptoms, as the Verticillium wilt symptoms were expanding, and with the reverse relationship, was noted in the Teresa microclone, whereas the least predictable intensity was in the Elsanta microclone. By analysing the interrelationships between the disease development rate, it was found that the greatest predictable increase in the rate of phytophthorosis development, with an increase in the rate of

Verticillium wilt development, occurred in the Feltar microclone. The greatest predictable increase in the rate of Verticillium wilt development with an increase in the phytophthorosis rate occurred in the Elsanta microclone, and the least predictable increase was in the Feltar microclone.

The available domestic and foreign literature provides no information on correlations between the susceptibility of the strawberry to *P. cactorum* and *V. dahliae*. Few research papers in the field of resistance breeding of this species mention several other important correlations. Shaw et al. [1996] reported a correlation between resistance and fruit firmness in strawberries, while no correlation was found between resistance and productivity. Shaw et al. [2005], in the case of Verticillium wilt, observed no correlation between the percentage of infected parent plants and the percentage of infection in plants formed from the stolons. Fang et al. [2011] noted significant correlations between strawberry crown diseases and plant dieback, as well as between root diseases and plant dieback. The increased incidence of strawberry mortality, as well as root diseases, was negatively correlated with the dry weight of the plants. The authors found no significant correlation between strawberry crown diseases and root diseases. Liang and Lin [2014] observed a significant correlation between susceptibility of the leaves and susceptibility of the fruit to mildew, whereas the coefficients of correlation between susceptibility and the fruit weight, length and width were not significant. As reported by Eikemo and Stensvand [2015], no correlation was found between the resistance to strawberry crown rot and resistance to leather rot of the fruit. Lynn et al. [2024] noted that leaf disease phenotypes were not genetically correlated with fruit disease phenotypes, which suggests that two different genetic mechanisms may control the disease resistance of leaves and fruit. Ukalska et al. [2006] reported that there is a phenotypic correlation between strawberry leaf spot and the density of the plants, as well as between the flowering time and the plants' susceptibility to powdery mildew.

The heritability of resistance for the strawberry has not been well documented [Pincot et al. 2020]. High heritability results, ranging from 75% to 97%, obtained in the present experiment, suggest that in the strawberry microclones tested, susceptibility to Verticillium wilt and phytophthorosis are traits highly heritable, *i.e.* strongly determined genetically, and readily passed on to offspring. The results indicated that only genotypes that are least susceptible to Verticillium wilt and phytophthorosis should be selected for breeding work aimed at increasing strawberry's resistance to these diseases. A similarly high heritability result for Verticillium wilt was obtained by Shaw et al. [1996]. In their experiment, the heritability coefficient value fell within the range of h^2_{bs} = 84–88%. Shaw et al. [2008] estimated that the heritability coefficient for phytophthorosis was 63%. This coefficient proved similar for strawberry resistance to mildew, as observed by Lifshitz et al. [2007] and Liang and Lin [2014]. Lifshitz et al. [2007] report that heritability with respect to mildew was 50%, whereas in an experiment by Liang and Lin [2014], it ranged from 66% to 68%.

The results obtained in the present study demonstrated the usefulness of *in vitro* culture selection for conducting more detailed research into genetic resistance mechanisms in strawberries. This approach supports the continued development of breeding efforts within this species.

CONCLUSION

The susceptibility of strawberry microplants to infection by *Verticillium dahliae* and *Phytophthora cactorum* varied, and the range and rate of disease development depended on the microclone studied. The similar course of phytophthorosis and Verticillium wilt observed in each microclone, confirmed by slight and mostly insignificant differences in the range and rate of development of both diseases, suggested the occurrence of similar mechanisms of genetic resistance to these pathogens. Moreover, the large genetic similarity (reaching over 80%) observed between submicroclones with similar susceptibility to both pathogens (*e.g.*, Elsanta and Teresa) also indicated the operation of similar genetic defense mechanisms in these varieties. High and positive correlations estimated for both the scope and the rate of development of pathogens in the analyzed microclones and the high value of the heritability coefficient of these traits additionally confirmed the activation of similar genetic defense mechanisms in plants in response to infection caused by *V. dahliae* and *P. cactorum*.

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FRUIT QUALITY OF NEW BLUE HONEYSUCKLE BERRY CULTIVARS AFTER SHORT-TERM STORAGE UNDER DIFFERENT CONDITIONS

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ABSTRACT

Lonicera caerulea L. (blue honeysuckle) is an edible species cultivated for the health properties of its fruit; however, fresh fruit has a short shelf life after harvest. Therefore, the present study aimed to extend the post-harvest shelf life of fresh honeysuckle fruits under controlled storage conditions. The quality of three Canadian blue honeysuckle cultivars was assessed after 7 and 14 days of storage in a controlled atmosphere (20% CO_2 and 5% O_2) (CA), modified atmosphere (MAP) in Xtend bags and air atmosphere (AA). The duration of storage conditions significantly affected the quality traits of blue honeysuckle berries. Extended storage time (14 days) generally had a negative effect on the quality of the tested fruit, especially when stored in air atmosphere. The berries stored in a controlled atmosphere showed the best quality, as evidenced by the highest firmness, the lowest weight loss, and the smallest percentage of rotten berries. The effect of storage conditions on the value of parameters such as soluble solids (SSC), titratable acidity (TA) or the SSC/TA ratio were often not observed. The respiration rate of fruits was usually independent of both the cultivar and storage conditions. Compared to other cultivars, Boreal Beauty fruits were characterized by a lower content of SSC, higher TA and a lower SSC/TA ratio, and lower polyphenol content. Fruits of the Boreal Blizzard showed the highest susceptibility to rot.

Keywords: *Lonicera caerulea* L., controlled atmosphere, modified atmosphere, fruit shelf life, Xtend bags

INTRODUCTION

Lonicera caerulea L. (commonly known as blue honeysuckle, haskap or honeyberry) belong to the family *Caprifoliaceae* and comprises approx. 250 species of plants native to the Northern Hemisphere [Dziedzic et al. 2020]. Alongside actinidia and cherry silverberry, blue honeysuckle belongs to the group of lesser known berries with a high potential for health promotion [Szot and Lipa 2012, Bieniek et al. 2017, Krupa et al. 2022, Garg et al. 2023]. Blue honeysuckle is generally cultivated in northern regions due to its exceptional cold hardiness. Some reports suggest that blue honeysuckle flowers can tolerate temperatures as low as –8 °C while the entire plants can survive temperatures down to –40°C [Hummer et al. 2012, Gasic et al. 2018]. In addition to its adaptation to cold climates, blue honeysuckle is also valued for its early harvest time relative to other berry crops. Blue honeysuckles ripen earlier than raspberries, blueberries, and strawberries [Leisso et al. 2021a]. Honeysuckle berries are rich in biologically active compounds, which is why they are highly valued by conscious consumers and the processing industry [Kaczmarska et al. 2014,

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Wang et al. 2018]. In addition to their interesting taste, the berries are known for their high content of anthocyanins and vitamin C, which have strong anti-inflammatory and antioxidant effects [Celli et al. 2014] and antidiabetic effect [Łysiak and Szot 2023]. The sugar profile of these fruits is dominated by glucose and fructose, with smaller amounts of sucrose and sorbitol. They also contain other valuable elements and compounds such as calcium, magnesium, fiber, iridoids and polyunsaturated fatty acids [Kalisz et al. 2023]. Honeysuckle fruits are distinguished by their high antioxidant activity [Martinez-Romero et al. 2007, Gawroński et al. 2020, Orsavova et al. 2022]. Also contain physiologically active phytochemicals including flavonoids and phenolic acids, which can contribute to the prevention of chronic diseases [Ochmian et al. 2012, Khattab et al. 2015]. Blue honeysuckle fruits have also been associated with a variety of therapeutic effects, such as lowering blood pressure, reducing the risk of heart attack, preventing osteoporosis and anemia, alleviating hyperactivity in children therapeutic effects for malaria and gastrointestinal disorders, and slowing the aging process [Gawroński et al. 2020]. As a newly introduced species, it is of interest to breeders. Self-pollinating cultivars are sought, as this is the key to obtaining high yields with good quality fruit [Parveze et al. 2024]. One of the most important features of new cultivars is their qualitative assessment and storage capacity. Fruit ripening is an irreversible developmental process that involves numerous biochemical and physiological changes resulting in a fruit with favorable organoleptic properties [Mac Kenzie et al. 2018, Gołba et al. 2020]. Blue honeysuckle fruits are characterized by high metabolic activity, which manifests itself through a high respiratory coefficient and transpiration. In addition, the high production of ethylene and their sensitivity to this gas make it difficult to store the fruit for a long time [Martinez-Romero et al. 2007]. As with many species, long-term storage is only possible by harvesting the fruit at the appropriate ripeness stage and ensuring careful handling to avoid damage that can increase ethylene production and accelerate ageing and reduce firmness [Martinez--Romero et al. 2007, Krupa et. al. 2023].Weight loss is one of the main factors limiting the storage life of berries [Horvitz 2017, Gawroński et al. 2020]. Fresh honeysuckle berries have a short storage period [Gerbrandt et al. 2020], of 7–10 days [Leisso et al. 2021b]. However using modified atmosphere packaging (MAP), such as Xtend bags, it is possible to prolong the storage period of blue honeysuckle berries up to 28 days [Blinnikova et al. 2021]. The storage of berries under refrigerated conditions (from –1°C to +2°C) preserves their quality by slowing natural metabolic processes such as respiration and transpiration [Dziedzic et al. 2020]. Given the high water content of the fruits, it is important to ensure adequate relative humidity (90–95%) during storage [Harb and Streif 2004].Very good results are obtained by storing fruits under the conditions of a modified atmosphere. The gas composition is determined in such a way as to slow down chemical changes, although it cannot stop them, because only then can the high biological value of the fruit be maintained [Harb et al. 2014, Bodbodak and Moshfeghifar 2016]. Berries tolerate high levels of carbon dioxide, but must not exceed 20% concentration while the minimum oxygen content is 2% [Leisso et al. 2022].

The purpose of the study was to evaluate the effect of storage conditions for 7 and 14 days in a modified atmosphere (MAP) and a controlled atmosphere (CA) on the quality of blue honeysuckle fruits.

MATERIAL AND METHODS

The research material consisted of three new blue honeysuckle (*Lonicera caerulea* L.) cultivars namely the Boreal Beauty, Boreal Beast, and Boreal Blizzard obtained from the University of Saskatchewan.

The fruit was harvested in a commercial plantation located in the south of Poland, about 30 km from Kraków at an altitude of 315 meters above sea level (50°17'31"N, 20°06'59"E). The plantation was established in 2018, on heavy, loess soil with a pH of 7–7.8 and a humus content of 2%, from plants propagated using the *in vitro* method. The plants were planted at a spacing of 4 × 1 m.

Under the climatic conditions of southern Poland, tested cultivars of blue honeysuckle cultivars generally begin their growing season in the third decade of March. In 2021, from the beginning of vegetation to fruit harvesting, that is, on June 25, the average temperature was 11.4 °C, and the total precipitation was 239.0 mm. In the following year 2022, during the same period, the average temperature was 10.7 °C,

and the total precipitation was 182.2 mm. The fruit was harvested on June 20.

The timing of harvesting is crucial to maintain high fruit quality during storage. The date of fruit harvest of each cultivar was determined on the basis of the color of the visual assessment of fruit (fully blue for all fruits) and the content of soluble solids (minimum 12.0%). This methodology is consistent with a study by Ochmian et al. [2012], who also collected blue honeysuckle fruits based on their color.

After delivery to the laboratory, the fruits were divided into three groups (four replicates, each containing about 300 g). All fruits were stored for 7 and 14 days at a temperature of 2 degrees Celsius (±0.5°C), using the following treatment:

1. Cold room (AA – air atmosphere) by keeping the relative humidity at 90–92%,
2. Controlled atmosphere cold room (20% CO_2 and 5% O_2) relative humidity as above,
3. Modified atmosphere pakages (MAP) in Xtend packaging (StePac L.A. Ltd. Johnson Mattey, Israel, USA)

The measurements and chemical analyses of the fruits were performed on a random sample with 40 fruits selected for each combination. Fruit firmness (N) was measured using a TA 500 Lloyd Texture Analyzer with a 6.35 mm diameter tip (AMETEK Test & Calibration Instruments; Fareham, Hampshire, United Kingdom). The soluble solid content SSC (°Brix) and the titratable acidity TA (% citric acid) were determined in the juice of blue honeysuckle berries, whose firmness had previously been measured.Soluble solids content was determined using an ATAGO PR 100 refractometer (ATAGO Co., Ltd.; Fukayashi, Saitama, Japan). Titratable acidity (TA) was determined by potentiometric titration at pH 8.1 with 0.1 N NaOH, using 5 mL of diluted juice in 100 ml of distilled water. Measurements were carried out using a CX 501 pH meter (ELMETRON; Zabrze, Poland) and then the soluble solids content to the titratable acidity ratio (SSC/TA). The fruit respiration rate (mg CO_2 kg^{-1} h^{-1}) was measured (using a sample of 25 blue honeysuckle berries from each combination) with an Air Tech 2500-P CO_2 analyzer(GAZEX; Warsaw, Poland). The polyphenol compounds in the extracts were determined on the reaction with the Folin–Ciocalteu reagent. The juice sample (0.25 mL) was mixed with 0.25 mL of 25% Na_2CO_3, 0.125 mL of the Folin–Ciocalteu reagent (Sigma–Aldrich, diluted twice with water prior to the analysis), 2.25 mL of water, and then incubated for 15 min. The absorbance was measured at 760 nm (JASCO V-530 UV–Vis spectrophotometer). The results were expressed as mg of gallic acid GAE per 100 mL. Furthermore, natural weight losses (%) were calculated based on the difference in fruit weight before and after storage and the percentage of fungal decay (%) was determined.

Data were analyzed using a two-way analysis of variance (ANOVA) implemented in Statistica software v. 13.3 (Tibco Software Inc., Palo Alto, CA, USA) with calculations conducted separately for each year and each date of fruits measurement and analysis (harvest, 7 and 14 day of storage). Values expressed as a percentages were transformed using the Bliss function ($y = \arcsin \sqrt{x}$). Tukey's HSD test was used to determine the significance of differences between mean values at a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of Leisso et al. [2021a] confirmed the involvement of ethylene in blue honeysuckle, but the evolution of CO_2 from detached fruits does not indicate classical climacteric ripening. These authors also suggest that determining the optimal harvest time for blue honeysuckle berries is challenging as the fruit darkens before reaching full maturity [Gerbrandt et al. 2020]. At harvest, depending on the cultivar, the ripeness stage and cultivation region, blue honeysuckle berries have a firmness ranging from 2.9 to 4.9 N [Dziedzic et al. 2020,Leisso et al. 2022], contain between 9.6 to 20.4°Brix SSC [Mac Kenzie et al. 2018, Dziedzic et al. 2020, Gerbrandt et al. 2020, Leisso et al. 2021a], with titratable acidity (TA) ranging from 1.8 to 4.4 % citric acid [Ochmian et al. 2012, Dziedzic et al. 2020, Gerbrandt et al. 2020, Leisso et al. 2021a], and the SSC/TA ratio ranging from 3.0 to high as 18.3 [Ochmian et al. 2012, Dziedzic et al. 2020, Gerbrandt et al. 2020, Leisso et al. 2021a]. Additionally blue honeysuckle berries were shown to have a high respiration rate, in the range of 208.8–310.8 mg CO_2 kg^{-1} h^{-1} [Dziedzic et al. 2020] or 1781.1–2463.4 nmol CO_2 kg^{-1} s^{-1} [Leisso et al. 2022]. According to Leisso et al. [2021a], the quality traits of the blue honeysuckle fruit at harvest showed

significant variability in terms of SSC, TA, SSC/TA ratio and firmness of the flesh.

The values of the quality parameters evaluated directly after harvest depended on the cultivar (Tab. 1). Boreal Beauty berries were found to be distinguished, compared to other cultivars, were distinguished by the highest firmness and acid content (TA), while on the other hand, they had the lowest extract content (SSC) and the lowest SSC/TA ratio. Leisso et al. [2022] also obtained a lower SSC content in Boreal Beauty fruits compared to other cultivars. The lowest SSC/TA ratio observed in Boreal Beauty berries indicated that they were less ripe and less sweet compared to other fruits. In addition, in the first year of the study, the respiration rate of Boreal Beauty fruits was higher than that of the other cultivars.

Blue honeysuckle berries are distinguished by their high content of phenol compounds [Rupasinghe et al.

Table 1. Fruit quality of blue honeysuckle berries directly after harvest

Year	Cultivars	Fruit firmness (N)	Soluble Solids Content (°Brix)	Titratable Acidity (% Citric Acid)	Ratio SSC/TA	Respiration Rate (mg CO_2 kg^{-1} h^{-1})
2021	Boreal Beauty	2.1 ±0.80c*	12.1 ±0.40a	2.74 ±0.12c	4.4 ±0.42a	101.9 ±25.74b
	Boreal Beast	2.0 ±0.61b	13.4 ±0.33b	2.27 ±0.10b	5.9 ±0.40b	51.3 ±18.95a
	Boreal Blizzard	1.8 ±0.49a	13.6 ±0.17b	1.40 ±0.05a	9.9±0.46c	35.4 ±7.62a
2022	Boreal Beauty	2.1 ±0.50b	12.8 ±0.26a	2.62 ±0.04c	4.9±0.16a	95.0 ±20.78a
	Boreal Beast	1.5 ±0.30a	16.4±0.50c	2.32 ±0.06b	7.0 ±0.17b	120.9 ±36.76a
	Boreal Blizzard	1.5 ±0.38a	15.0 ±0.15b	1.57 ±0.03a	9.6 ±0.28c	104.0 ±41.57a

*Means followed by the same letter within a column, for each year, do not differ significantly at $p \leq 0.054$

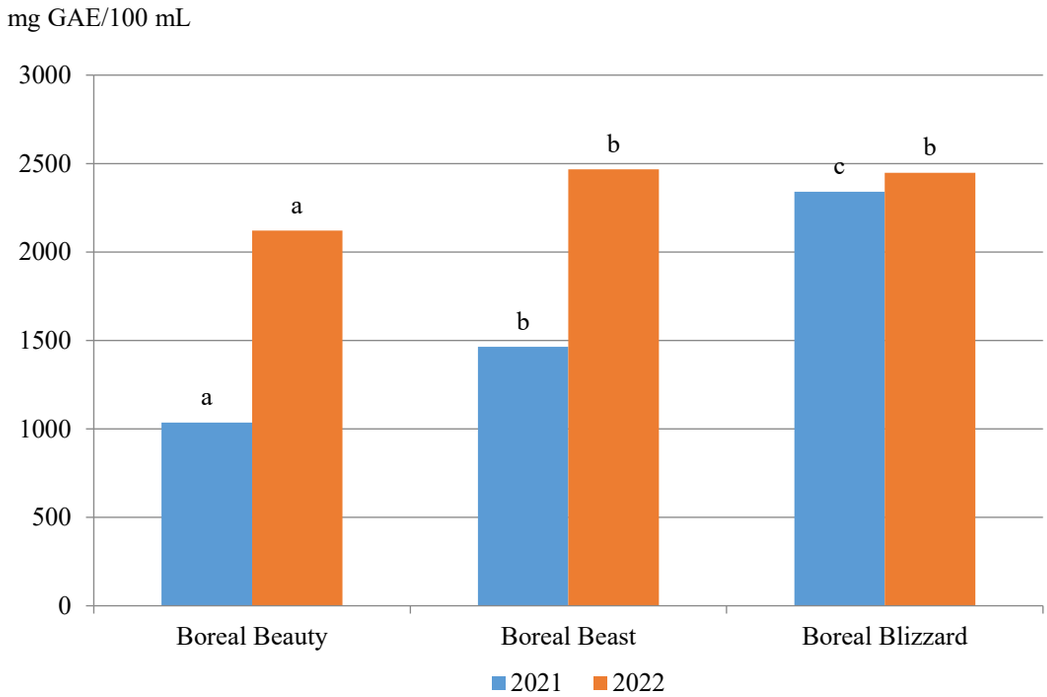

Fig. 1. Total polyphenol content (mg GAE/100 mL) of blue honeysuckle berries directly after harvest

2018]. According to Shevchuk et al. [2022], the Boreal group of cultivars has a higher polyphenol content compared to the other cultivars analyzed. The polyphenol content determined after harvest varied significantly by cultivar (Fig. 1). In the first year of the study, the Boreal Blizzard contained more polyphenols than berries of the other cultivars. In the following year, the polyphenol content of Boreal Blizzard fruits was higher compared to that determined in Boreal Beauty.

The interaction between experimental factors, such as variety and storage conditions, generally had a significant impact on the values of quality parameters (Tab. 2–5). Only the respiration rate of berries measured in 2021 after 7 days of storage (Tab. 2), and in 2022 after 14 days of storage (Tab. 5), was not influenced by the experimental factors. Firmness and weight loss are among the most important parameters that characterise the quality of berries after storage [Dziedzic et al. 2020]. Equally important components of fruit quality are the total content of soluble solids (SSC) and titratable acidity (TA) [Szot and Lipa 2012, Gerbrandt et al. 2020]. During storage, the firmness of blue honeysuckle berries gradually decreases [Mac Kenzie et al. 2018, Dziedzic et al. 2020, Leisso et al. 2021b, Leisso et al. 2022]. In the present study, at each date of measurement of the firmness of the fruit, the berries of a given cultivar stored under AA conditions consistently exhibited lower values compared to those

Table 2. Effect of cultivars and storage conditions on the quality of blue honeysuckle berries after 7 days of storage in 2021

Cultivars	Storage conditions	Fruit firmness (N)	Soluble Solids Content (°Brix)	Titratable acidity (% citric acid)	Ratio SSC/TA	Respiration rate (mg CO_2 kg^{-1} h^{-1})
Boreal Beauty	AA	1.5 ±0.12a*	10.8 ±0.10 b	2.24 ±0.04bc	4.8 ±0.11b	145.1 ±34.99a
	MAP	1.9 ±0.14 e	11.2 ±0.15 b	2.31 ±0.05 c	4.8 ±0.18b	104.8 ±29.40 a
	CA	2.0 ±0.15ef	10.1 ±0.16a	2.47±0.03 d	4.1 ±0.16a	117.5±49.61 a
Boreal Beast	AA	1.6 ±0.13bc	12.4 ±0.20 c	2.15 ±0.06 b	5.8 ±0.11 c	186.2 ±52.32a
	MAP	1.8 ±0.11d	13.8 ±0.14d	2.26 ±0.03bc	6.1 ±0.19c	102.4 ±14.22a
	CA	2.0 ±0.15 f	12.8 ±0.17c	2.25 ±0.04 bc	5.7 ±0.14c	149.6 ±29.01a
Boreal Blizzard	AA	1.5 ±0.14ab	13.8 ±0.21d	1.22 ±0.04 a	11.3 ±0.36 e	143.0 ±36.12a
	MAP	1.7 ±0.11cd	14.1 ±0.12d	1.25 ±0.05a	11.2 ±0.41de	107.9 ±25.43a
	CA	1.7 ±0.14cd	13.7 ±0.28d	1.29 ±0.06a	10.6 ±0.32 d	109.6 ±25.85a

*Means followed by the same letter within a column do not differ significantly at $p \leq 0.05$

Table 3. Effect of cultivars and storage conditions on the quality of blue honeysuckle berries after 14 days of storage in 2021

Cultivars	Storage conditions	Fruit firmness (N)	Soluble Solids Content (°Brix)	Titratable acidity (% citric acid)	Ratio SSC/TA	Respiration rate (mg CO_2 kg^{-1} h^{-1})
Boreal Beauty	AA	1.3 ±0.13a*	10.9 ±0.25b	2.52 ±0.05 c	4.3 ±0.18 a	159.7 ±48.83 bc
	MAP	1.6 ±0.10c	11.2 ±0.10b	2.67 ±0.08c	4.2 ±0.12a	123.2 ±18.97 abc
	CA	1.8 ±0.16d	10.3±0.15 a	2.60 ±0.09c	4.0 ±0.13a	120.3 ±23.89 abc
Boreal Beast	AA	1.4 ±0.12ab	15.1 ±0.26 ef	2.20 ±0.02b	6.9 ±0.22b	156.8 ±40.11 bc
	MAP	1.5 ±0.12bc	14.8 ±0.12de	2.24 ±0.04b	6.6±0.15 b	110.9 ±29.32 abc
	CA	1.9±0.15 d	15.6±0.10 f	2.25 ±0.09b	7.0 ±0.28b	49.8 ±14.66 a
Boreal Blizzard	AA	1.3 ±0.15 a	14.3 ±0.15cd	1.15 ±0.08a	12.4 ±0.32d	187.0 ±30.92c
	MAP	1.4 ±0.10ab	13.9 ±0.20c	1.21 ±0.05a	11.5 ±0.35cd	91.0 ±20.18abc
	CA	1.5 ±0.14bc	14.4 ±0.21cd	1.29 ±0.01a	11.2 ±0.18c	68.0 ±20.30ab

*Means followed by the same letter within a column do not differ significantly at $p \leq 0.05$

Table 4. Effect of cultivars and storage conditions on the quality of blue honeysuckle berries after 7 days of storage in 2022

Cultivars	Storage conditions	Fruit firmness (N)	Soluble Solids Content (°Brix)	Titratable acidity (% citric acid)	Ratio SSC/TA	Respiration rate (mg CO_2 kg^{-1} h^{-1})
Boreal Beauty	AA	1.4 ±0.11d*	12.1 ±0.17a	2.17 ±0.02b	5.6 ±0.35a	143.9 ±24.79a
	MAP	1.6 ±0.11e	12.1 ±0.32a	2.23 ±0.04b	5.4 ±0.17a	110.5 ±33.11a
	CA	1.8 ±0.14g	12.2 ±0.38a	2.22 ±0.08b	5.5 ±0.43a	81.8 ±11.55a
Boreal Beast	AA	1.3 ±0.12d	15.8 ±0.42cd	2.02 ±0.09b	7.8 ±0.44b	119.0 ±15.29a
	MAP	2.1 ±0.11h	15.8 ±0.50cd	2.13 ±0.03b	7.4 ±0.25b	69.4 ±13.63a
	CA	1.7 ±0.11f	16.1 ±0.42d	2,09 ±0.07b	7.8 ±0.27b	86.7 ±23.88a
Boreal Blizzard	AA	1.0 ±0.12a	14.4 ±0.21b	1.25 ±0.01a	11.5 ±0.13c	113.5 ±40.90a
	MAP	1.1 ±0.11b	14.7 ±0.31bc	1.26 ±0.03a	11.6 ±0.14c	104.9 ±48.70a
	CA	1.2 ±0.13c	14.1 ±0.40 b	1.30 ±0.07a	10.9 ±0.20 c	91.8 ±30.73a

*Means followed by the same letter within a column do not differ significantly at $p \leq 0.05$

Table 5. Effect of cultivars and storage conditions on the quality of blue honeysuckle berries after 14 days of storage in 2022

Cultivars	Storage conditions	Fruit firmness (N)	Soluble Solids Conten t (°Brix)	Titratable acidity (% citric acid)	Ratio SSC/TA	Respiration rate (mg CO_2 kg^{-1} h^{-1})
Boreal Beauty	AA	1.4 ±0.20b*	11.6 ±0.42a	2.11 ±0.03c	5.5 ±0.13a	148.0 ±41.46a
	MAP	1.6 ±0.10 cde	12.4 ±0.45a	2.32 ±0.06d	5.3 ±0.16a	112.1 ±25.19a
	CA	1.7 ±0.14ef	12.3 ±0.38a	2.30 ±0.01d	5.4 ±0.24a	64.6 ±16.12a
Boreal Beast	AA	1.6 ±0.12cde	15.5 ±0.16c	1.82 ±0.03b	8.5 ±015b	129.6 ±38.78a
	MAP	1.7 ±0.13ef	15.8 ±0.26c	1.91 ±0.01b	8.3 ±0.21b	107.5 ±31.93a
	CA	1.9 ±0.12f	15.9 ±0.28c	1.92 ±0.08b	8.3 ±0.24b	66.7 ±10.01a
Boreal Blizzard	AA	0.8 ±0.11a	14.0 ±0.21b	1.24 ±0.03a	11.3 ±0.12c	140.8 ±41.30a
	MAP	1.5 ±0.16bc	14.2 ±0.10 b	1.30 ±0.03a	10.9 ±0.16c	99.8 ±23.75a
	CA	1.6 ±0.20 cde	13.7 ±0.23b	1.30 ±0.02a	10.5 ±0.12c	69.8 ±18.39a

*Means followed by the same letter within a column do not differ significantly at $p \leq 0.05$

stored in CA and frequently also compared to the berries stored in MAP. Dziedzic et al. [2020] demonstrated that the firmness of blue honeysuckle berries stored under controlled atmosphere conditions was well preserved.

In both years of the study, the impact of cultivar on the soluble solids content (SSC) of the berry juice measured after storage was significant. The berries of the cultivar Boreal Beauty cultivar were shown to have a lower SSC content compared to the other cultivars. On the contrary, the storage conditions of the fruits did not always have a significant effect on the value of the described trait. The culture has previously shown a greater effect of cultivar than the storage conditions on the SSC content of blue honeysuckle berries by Dziedzic et al. [2020]. This relationship was recorded, among others, in 2021 for the cultivar Boreal Blizzard after 7 days of storage (Tab. 2), and in 2022 for the fruits of all studied blue honeysuckle cultivars after both 7 and 14 days of storage (Tabs 4, 5). According to Leisso et al. [2021b], the SSC content in blue honeysuckle berries remained relatively stable during the postharvest period. In our study, we observed a different relationship; in the first year, the SSC content of

the berries during storage was generally higher than at harvest, while in the second year, it was lower.

The titratable acidity (TA) of blue honeysuckle berries decreases during storage [Mac Kenzie et al. 2018, Dziedzic et al. 2020, Leisso et al. 2021, Leisso et al. 2022]. Similarly to SSC, the TA of blue honeysuckle berries was significantly dependent on the cultivar. In general, regardless of storage conditions, the fruits of the Boreal Beauty had the highest TA values. In contrast, the lowest TA was observed for the Boreal Blizzard cultivar. On the other hand, the effect of storage conditions on the TA of the fruits was observed only in the Boreal Beauty. In 2021, after 7 days of storage, berries from CA exhibited higher TA values compared to those stored under MAP and AA conditions (Tab. 2). In the following year, after 14 days of storage, the berries from the CA and MAP treatments had a higher TA content than those stored in AA (Tab. 5). In the present study, we did not observe a significant decrease in TA during the storage of blue honeysuckle berries, similar to that reported by Leisso et al. [2021b].

The SSC/TA ratio increases during berry storage [Leisso et al. 2021b], which indicates favorable changes in consumer perception of the fruits [Harker et al. 2002, Jayasena and Cameron 2008]. Regardless of the storage conditions, the SSC/TA ratio for the cultivar Boreal Beauty was significantly lower at each time point compared to the values calculated for the remaining varieties. The highest value of the described trait was consistently observed in the fruits of the cultivar Boreal Blizzard fruit (Tabs 2–5). The low SSC/TA ratio associated with the cultivar Boreal Beauty was clearly associated with its lower perceived sweetness when consuming the berries, while the sweetest fruits had the cultivar Boreal Blizzard. The cultivar, in combination with fruit storage conditions, had a significant impact on the SSC/TA ratio only during the first year of the study (Tabs 2, 3). After 7 days of storage, the fruits of the cultivar Boreal Beauty stored under CA conditions exhibited a lower value of the described indicator compared to those of MAP and AA. For the cultivar Boreal Blizzard, the fruits stored in CA showed a lower value of the SSC/TA ratio compared to those stored in AA. This relationship was observed after both 7 and 14 days of storage.

Blue honeysuckle berries are characterized by a high respiration rate (Tabs 1–5). According to Dziedzic et al. [2020], blue honeysuckle berries are non-climacteric fruits, therefore, are not characterized by increased respiration.

Respiration is, however, necessary to meet the energy requirements necessary to maintain all metabolic processes. During this process, stored carbohydrates, lipids, and organic acids are broken down [Fonseca et al. 2002]. The present study demonstrated that the respiration rate of blue honeysuckle berries was generally not influenced by either the cultivar or the storage conditions. Leisso et al. [2022], on the other hand, suggested that the cultivar affected the CO_2 production. Only in 2021, after 14 days of storage, the value of this parameter depended on the interaction between cultivar and storage conditions (Tab. 3). The respiration rate of Boreal Beast fruits (49.8 mg CO_2 kg^{-1} h^{-1}) and Boreal Blizzard fruits (68.0 mg CO_2 kg^{-1} h^{-1}) fruits stored in CA was significantly lower compared to Boreal Blizzard fruits from the AA combination (187.0 mg CO_2 kg^{-1} h^{-1}). Fonseca et al. [2002] reported that respiration slowed due to reduced O_2 availability resulting from reduced overall metabolic activity. A beneficial effect of storing blue honeysuckle fruits in CA on the reduction of respiration rate was previously demonstrated by Dziedzic et al. [2020].

The polyphenol content of the blue honeysuckle berries decreased successively during storage (Figs 2 and 3). Polyphenols are sensitive to oxidative stress and enzymatic activity, which can lead to their degradation during storage [Zhang et al. 2021]. Fruits stored under CA and MAP conditions are slower to carry out metabolic processes, which is one of the factors in oxidative stress. The fruits of the tested cultivars stored under MAP and CA conditions generally contained more polyphenols compared to those under AA conditions. The content of polyphenols during storage is a species and cultivar feature that is largely influenced by the gas composition, especially the concentration of carbon dioxide [Khorshidi et al. 2011, Harb et al. 2014, Dziedzic et al. 2020]. A significant effect of storage conditions on the described trait was not shown only for the Boreal Beast cultivar in the first year of the study after 7 days of storage. On the other hand, after 14 days of storage, the same content of polyphenols was characterized by fruits of Boreal Beast and Boreal Blizzard cultivars with AA and MAP. This relationship was also noted in the second year of the study

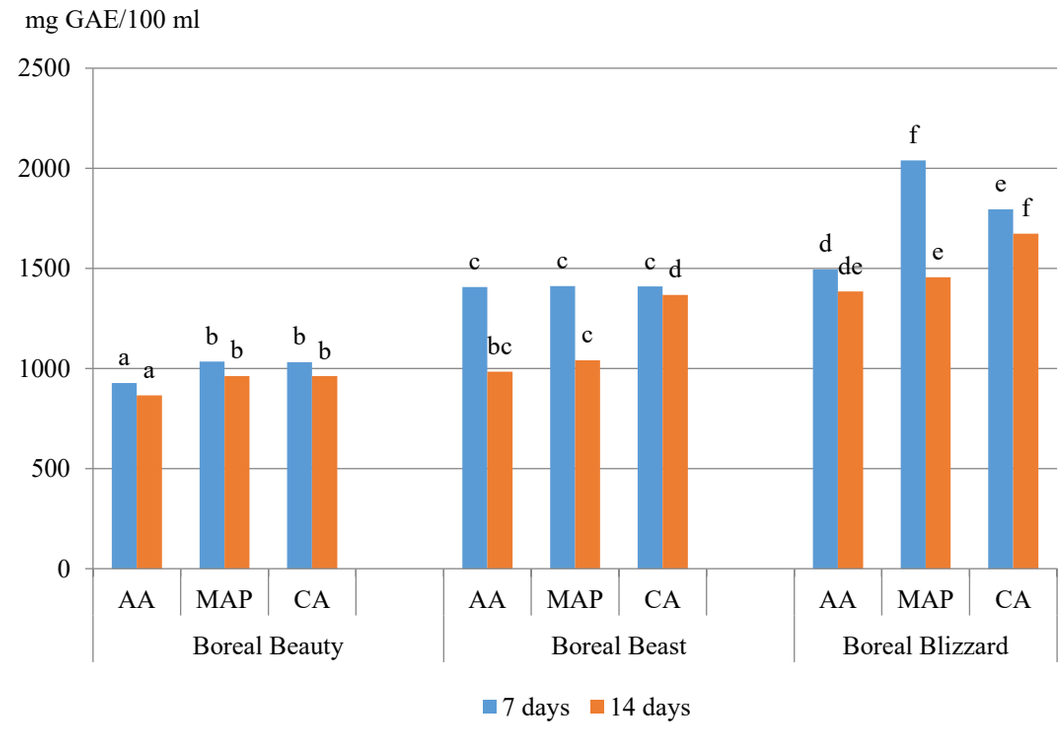

Fig. 2. Total polyphenol content (mg GAE/100 mL) of blue honeysuckle berries after 7 and 14 days of storage in 2021

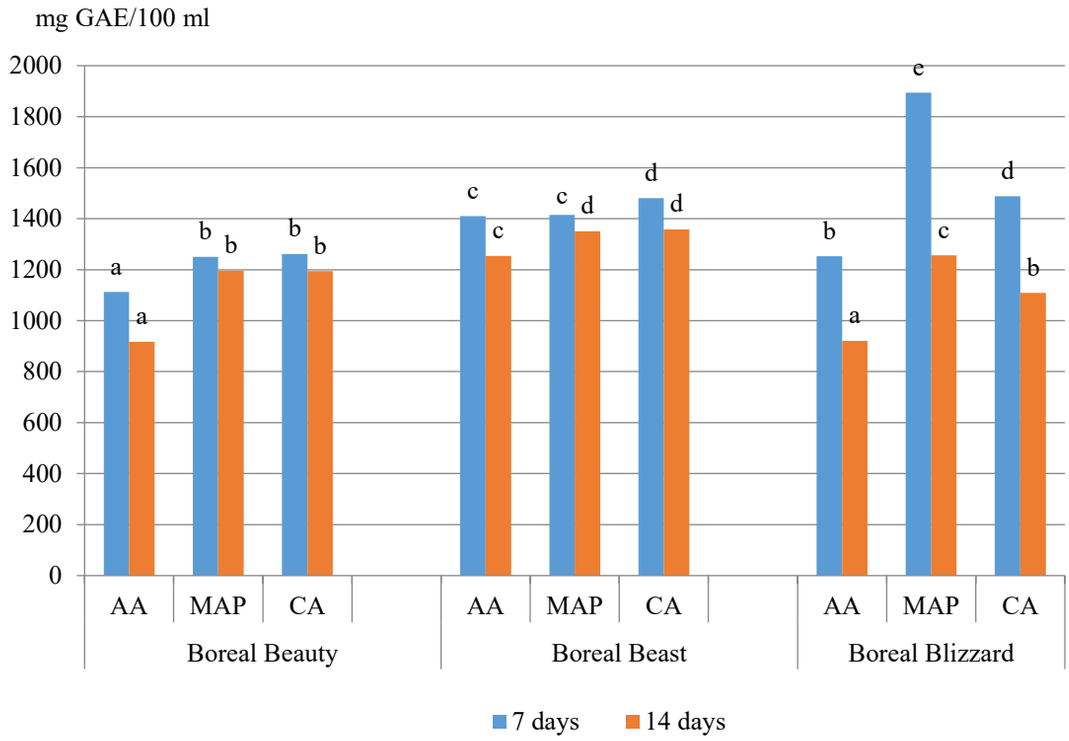

Fig. 3. Total polyphenol content (mg GAE/100 mL) of blue honeysuckle berries after 7 and 14 days of storage in 2022

for the Boreal Beast cultivar after 7 days of storage. Boreal Beauty fruits generally had a lower polyphenol content compared to Boreal Beast and Boreal Blizzard fruits determined after 7 and 14 days of storage. The exceptions to this rule were similar polyphenol content in Boreal Beauty fruit stored in MAP and CA and Boreal Beast fruit under AA conditions (2021 after 7 days of storage) and in 2022 after 14 days of storage in Boreal Beauty and Boreal Blizzard fruit stored in AA and CA.

The results obtained in our study showed that the weight losses of berries of all cultivars stored in CA were always lower compared to those measured in fruits from the AA environment. We have reported very similar relationships in our earlier study [Dziedzic et al. 2020]. The only exception was the weight loss of Boreal Beast fruits recorded in 2022 after 7 days of storage. For fruits stored in MAP bags, the parameter value of the discussed was often statistically the same as under fruits stored under AA conditions. However, Blinnikova et al. [2021] demonstrated that storage of blue honeysuckle berries in MAP reduced fruit weight loss by 1.5 times compared to storage under AA conditions. In addition to high respiration rates, blue honeysuckle fruits are also characterized by significant transpiration [Martinez-Romero et al. 2007], which can lead to substantial weight loss in stored berries (Figs 4–7).

Fruit decay during storage (Figs 4–7) is another source of loss and a limiting factor for the storage time of blue honeysuckle berries [Leisso et al. 2021]. The main pathogenic organism that causes the rotting of berries is grey mould (*Botrytis cinerea*) [Wan et al. 2021]. The value of this characteristic was significantly dependent on the interaction between the cultivar and storage conditions of fruit. The storage of berries in CA always resulted in a lower percentage of rotten fruit compared to AA. MAP conditions tended to limit fruit rot more effectively than AA. According to Blinnikova et al. [2021], the storage of blue honeysuckle berries in MAP bags significantly reduced the number of rotten fruits compared to the control (AA). In the present study, extending the storage period of Boreal Beauty fruits did not result in a higher percentage of rotten berries, compared to the other two cultivars. Additionally, it was found that the cultivar Boreal Blizzard was more susceptible to fruit rot.

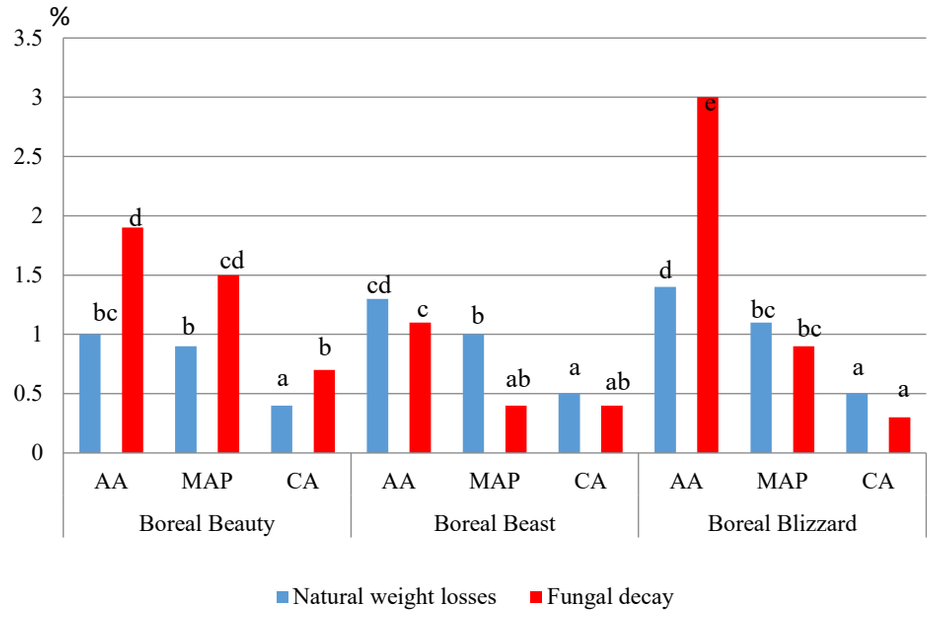

Fig. 4. Effect of cultivars and storage conditions on the natural weight losses (%) and fungal decay (%) of blue honeysuckle berries after 7 days of storage in 2021

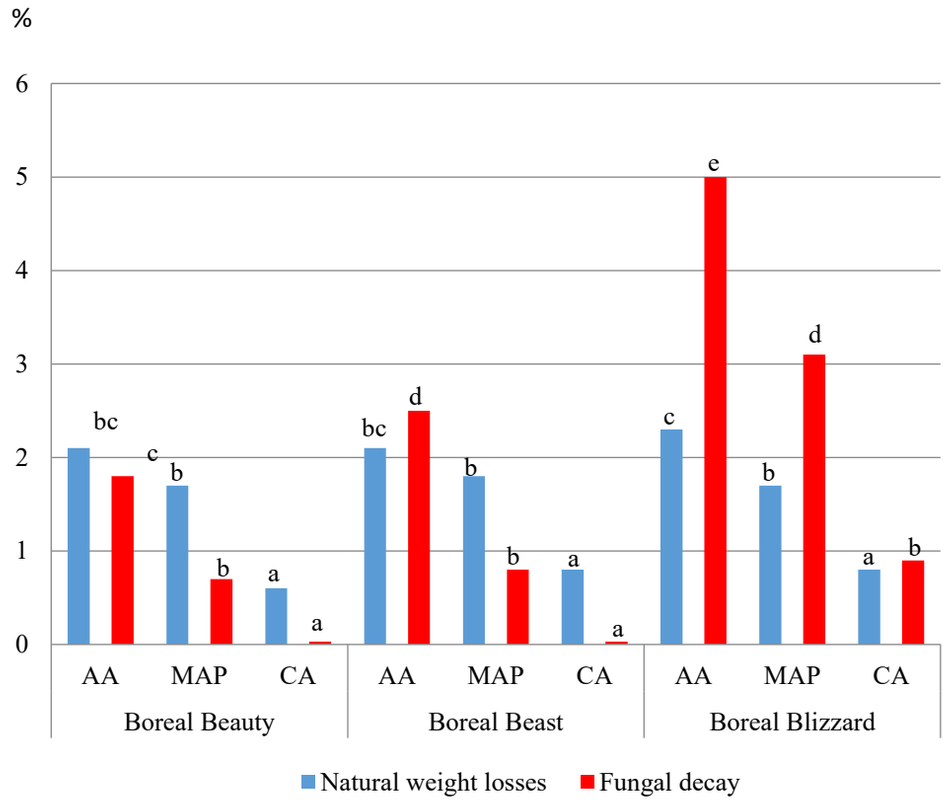

Fig. 5. Effect of cultivars and storage conditions on the natural weight losses (%) and fungal decay (%) of blue honeysuckle berries after 14 days of storage in 2021

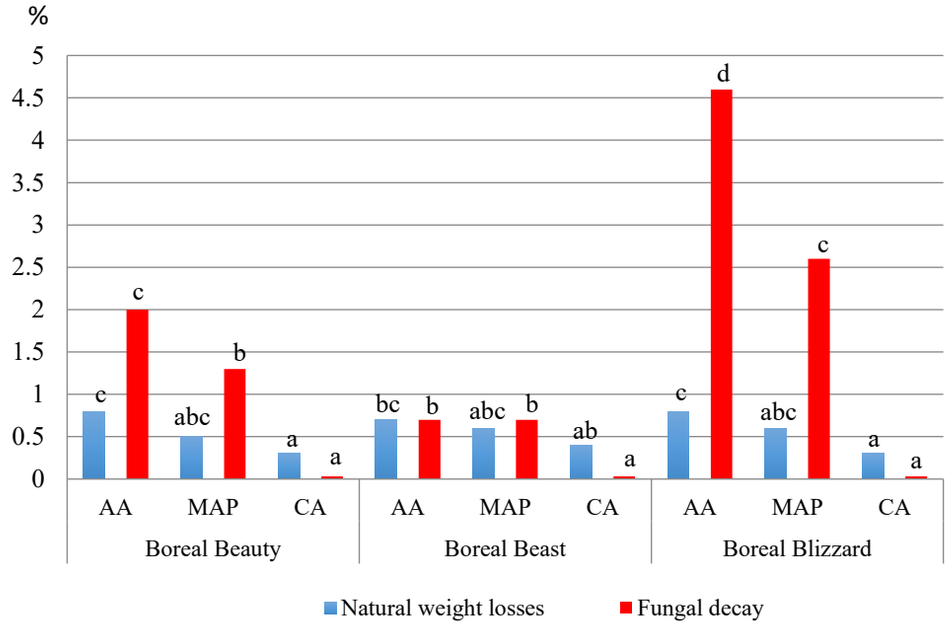

Fig. 6. Effect of cultivars and storage conditions on the natural weight losses (%) and fungal decay (%) of blue honeysuckle berries after 7 days of storage in 2022

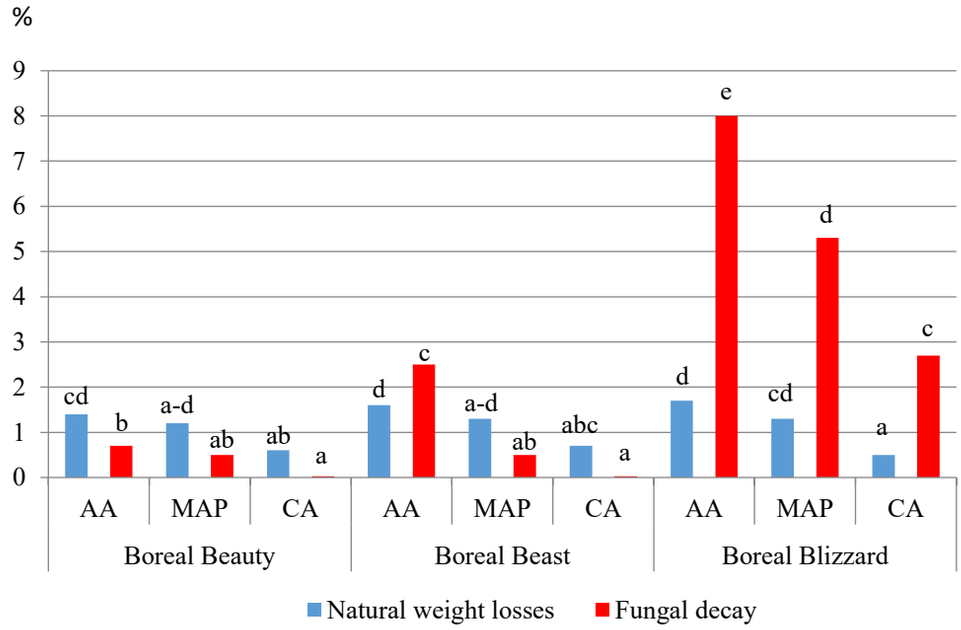

Fig. 7. Effect of cultivars and storage conditions on the natural weight losses (%) and fungal decay (%) of blue honeysuckle berries after 14 days of storage in 2022

CONCLUSIONS

Extended storage time (14 days) most often had a negative effect on the quality of the tested fruit, especially when stored in air atmosphere. Storing the berries in a controlled atmosphere guaranteed their best quality, as evidenced by the highest firmness, the lowest weight loss and the lowest percentage of rotten berries. The effect of storage conditions on the value of parameters such as soluble solids-SSC, titratable acidity-TA or the SSC/TA ratio were often not observed. The respiration rate of fruits was usually independent of both the cultivar and storage conditions. Compared to other cultivars, the fruit of the Boreal Beauty cultivar was characterized by a lower content of soluble substances SSC, a higher titratable acidity TA, and a lower SSC/TA ratio, and lower polyphenol content. The fruit of the Boreal Blizzard cultivar turned out to be the most susceptible to rot, therefore they can be stored longer (14 days) only in a controlled atmosphere. All storage conditions (AA, MAP and CA) ensure high quality of blue honeysuckle berries when stored for 7 days. However, when the storage period is extended to 14 days, only the CA conditions ensure the preservation of high fruits quality. Results of the presented research can be used in practice to create optimal conditions for short-term storage of blue honeysuckle berries and to ensure appropriate transport conditions for these fruits in order to maintain their high quality and shelf life.

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MICROBIAL AND PHYSICO-CHEMICAL RESPONSES OF THE SOIL TO INTENSIVE ONION AND PEPPER CROPPING

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ABSTRACT

Vegetable cropping systems are high-input and generally require large quantities of fertilization, protection, frequent irrigation, and repeated tillage operations. Consequently, an increase in vegetable production may have serious impact on soil health and functions. The aim of the study was to assess microbiological, chemical and physical indicators of soil fatigue in two of the most intensive vegetable crops in Poland: onions and peppers, to identify which cultivation practices are most responsible for the adverse changes. The results have shown, that the most reliable indices in cultivation of these vegetables occurred dehydrogenase activity, organic matter content and soil physical properties. The other studied parameters such as pH, nutrients availability and microbial abundance seem to be less sensitive factors. In all soils, where the onion and pepper were produced, the dehydrogenase activity was significantly lower as compared to non-cultivated soil. It corresponded with reduced content of organic matter. In onion production numerous runs by agricultural machinery during field operations lead to soil compaction, breakdown of its structure and organic matter reduction. Moreover, poor crop rotation and low surface coverage with vegetation accelerate these effects and deteriorate the biological functioning of the soil. In turn, in pepper cultivation, monoculture with high mineral fertilization, cause soil acidification and adverse effect on microorganisms, decreasing their activity, but increasing the proportion of fungi in microbial community. Intense mineral input, resulting in high concentration of nutrients in soil, may be a reason of reduced organic carbon content, despite application of organic manures.

Keywords: onion, pepper, dehydrogenase, organic matter, soil structure, microorganisms, nutrients

INTRODUCTION

Soil is a natural, non-renewable resource, which has fundamental functions to our existence as a source of food and energy. Decomposition of organic matter and circulation of nutrients, essential for the continuity of life, take place in the soil. It also plays an important role in climate regulation e.g. through carbon sequestration and water retention. However, in the 21st century, the progressive degradation of soils is a serious problem. Intensive agriculture cause a strong pressure on soil environment and negatively affects its functioning. In consequence, soil is subject to accelerated erosion, compaction, acidification, salinization,

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decline in organic matter and depletion of organic carbon reserves, elemental imbalance and loss of nutrients, loss of biodiversity and accumulation of soil-borne pathogens [Lal 2015, Bonanomi et al. 2016, Bayata 2024]. According to global estimates, about 38% of soils have been degraded, including those used for agricultural purposes [Goździewicz-Biechońska 2018], and at least 60–70% of soils in the European Union [Midler 2022, European Environment Agency 2024]. Changes to cropping systems are required to prevent these adverse trends. In order to do this, however, it is necessary to know which factors have a decisive impact on soil health in specific crops.

In the food supply chain vegetables play very important role. Sales of fresh produce – vegetables and fruits, has increased dramatically (by 30%) in the past few decades [Stea et al. 2020], as their consumption is intensively promoted worldwide to prevent many civilisation's diseases. However, growing vegetables is demanding in terms of nutrition, protection and irrigation. In spite of improved varieties, the pressure of pathogens and pests requires systematic control, and achieving satisfactory yields encourages farmers to use intensive fertilization and agro-technical practices with heavy equipment. This intensification of production leads to environmental pollution, residual toxicity towards microorganisms and humans, development of plant pathogen resistance and biodiversity loss [De Corato 2020], which results in serious crop problems and losses that farmers face for years. In Poland, vegetable producers complain that they have to apply more and more fertilizers and pesticides to achieve satisfactory yields, which exacerbates the detrimental processes in the soil.

Onion and pepper are the crops, which production is very intensive, with high input of chemicals and limited crop rotation. Onion occupies the first place in Poland in terms of the area of vegetable cultivation. The annual production in 2024 was around 700,000 tonnes. After the Netherlands and Spain, Poland is the third producer of onion in the EU, and the share of our country in the EU production of onion is about 10% [Statistics Poland 2024]. Pepper in Poland is the third vegetable grown under cover after tomatoes and cucumbers. The specificity of this crop lies in the fact, that the peppers are grown in plastic tunnels, in the soil, on the same site for up to a dozen years. In both cases, the production is associated with many difficulties, and growing conditions are getting worse. In order to try to solve these problems, it is necessary to know which soil parameters have been most severely altered by these crops.

Numerous chemical, physical, microbiological and biochemical indicators are used to asses soils condition [Bastida et al. 2008]. Among them pH, nutrients availability, soil organic matter (SOM) content and soil physical indices such as bulk density, soil porosity and field water capacity are considered as important for soil function [Chaudhry et al. 2024, Kahsay et al. 2025]. Of great importance are also microbial indices [Raiesi and Behesthi 2015]. Soil microorganisms plays an important role in organic matter decomposition and govern the balance of carbon cycling between SOM and atmospheric C pool, and microbial biomass contributes to SOM [Anthony et al. 2020]. Microorganisms are also responsible for nutrient cycling, soil structure building, promotion of plant growth and suppression of pathogens [Jacobsen and Hjelmsø 2014, Wolińska et al. 2018, Mayer et al. 2019]. The diversity of their metabolic activities makes them participate in practically all processes and stages of nutrient transformations in the soil [Bonanomi et al. 2016, Mahala et al. 2020]. According to Trivedi et al. [2016], changes in microbial populations and activity can precede detectable changes in soil physico-chemical properties, providing an early sign of soil degradation. Populations of bacteria, actinomycetes, fungi and soil enzyme activities are still the most commonly studied indicators in order to assess the performance of various soil management techniques [Tian et al. 2015, Wolińska et al. 2018]. Essential microbial function of processing and acquisition of nutrients from organic matter, requires the activity of extracellular enzymes [Raiesi and Behesthi 2015, Qu et al. 2020]. Among the soil enzymes, the activity of dehydrogenases (DHA) provides information about the biologically active microbial population in the soil [Gil-Sotres et al. 2005].

The aim of this study was to assess the condition of soils in intensive production of onion and pepper, as an attempt to try to solve the problems with soil degradation in cultivation of these vegetables. The evaluation was based on microbiological, chemical and physical indices of soil condition.

MATERIALS AND METHODS

Origin and sampling of soil

Soil samples were collected from Polish farms situated mainly in central Poland, where intensive, long-term vegetable cultivation is carried out. The farms for sampling were selected based on crop history, where in many cases the vegetables were grown in monoculture over several years. The best example is the cultivation of pepper in the soil, in PE film tunnels, on the same site for several years (even for 20 years). The crops were treated with intensive mineral fertilization, chemical plant protectants and mechanical cultivation using heavy equipment (except for peppers in tunnels). Soil samples were taken in the middle of vegetation period for crop plants. At the same time, comparative samples were taken from fallow soils in close proximity to the agricultural soils. Four combined soil samples were taken for each site. One combined sample, comprising approx. 2 kg of soil, was collecting from a randomly selected area of 25 m^2 (square 5 × 5 m). The sub-samples were taken with a Egner's tool from a depth of about 0–20 cm. These samples were taken for microbiological, chemical and biochemical analyses. From the same areas, the soil samples were collected to evaluate physical soil parameters using four stainless cylinders (diameter 5 cm, height 5 cm), closed on both sides with a tightly fitting plastic caps.

In total, arable and fallow soil samples (in four replicates) were taken from 28 farms: 18 from onion crops, 10 from pepper crops. During the farms visits, the farmers were asked to fill a questionnaire, in which the information about fertilization practices (the type of fertilizers used and time of their application) and on the crop rotation systems were collected.

Microbiological analyses

All combined soil samples were thoroughly mixed and sieved to remove stones and big particles. Then, 10 g subsamples were added to 100 ml 0.85% NaCl water solution and mixed on a rotary shaker for 20 min. The soil dilution plating method on selective media was used to quantify the population of selected groups of microorganisms. Total bacteria, including actinomycetes, were determined on soil extract medium [Dhingra and Sinclair 1995]. The number of fluorescent *Pseudomonas* was determined on the S1 medium under UV light [Gould et al. 1985]. Rose Bengal medium [Martin 1950] was used to enumerate fungi and yeasts. The number of spore-forming bacteria was determined on 10% trypticase soy agar medium (TSA), after pre-heating of the soil suspension for 10 minutes at 80 °C. Copiotrophs and oligotrophs were evaluated according to Hattori and Hattori method [1980]. The number of microorganisms was expressed as a $\log_{10}$ of colony forming units (cfu) g^{-1} of soil dry weight.

Enzyme dehydrogenases activity

Dehydrogenases activity (DHA) was measured according to Cassidy procedure described by Alef and Nannipieri [1995] and Brzezińska and Włodarczyk [2005]. Briefly, 3 g of sieved soil was placed in the 15 ml dark tube to restrict light, and added successively with: 1.8 ml of sterile deionized water, 600 µl of 1% glucose suspension and 600 µl of 3% water solution of 2,3,5-triphenyltetrazolium chloride (TTC). The mixture was incubated for 24 hours at 30 °C in the darkness. During incubation TTC was reduced enzymatically to water-insoluble TPF (1,3,5-triphenylformazane). The reaction was stopped by addition 12 ml of 96% ethanol, and the mixture was agitated for next hour in dark. Then, the samples were centrifuged (12 000 rpm/min.) for 8 minutes, at 4 °C. The supernatant was used for spectrophotometric measurements of TPF concentration at λ = 485 nm, with the use of spectrophotometer (UVLINE 9600). The obtained values were related to the standard curve (with known TPF concentration). Finally, the mean dehydrogenases activity was expressed in units of dehydrogenases activity, as the amount of TPF produced by 1 gram of soil during 24 hours (µg TPF/g s.d.w. 24 h) [Casida et al. 1964].

Chemical analyses of the soil

Sieved soil samples were subjected to chemical analysis. Total nitrogen was determined through mineralization in concentrated sulfuric acid [Walinga et al. 1995]. A Kjeldahl digestion kit and unit (Vapodest, Gerhardt GmbH, Konigswinter, Germany) were used to determine total nitrogen (N) content by titration. Macro- and micronutrients (P, K, Ca, Mg, Na, S, Fe, Mn, Cu, Zn, B) were isolated by the microwave digestion in the concentrated nitric acid, in a closed system

[Ostrowska et al. 1991]. Qualitative and quantitative analyses of the above elements were done by the plasma spectrometry, atomic absorption spectroscopy and colorimetric methods called "segment flow system" [Antweiler et al. 1996]. The pH was measured in 1 N KCl in air-dried soil, using a 1 : 5 v/v soil suspension.

Soil physical properties measurements

The physical properties of the soils were determined according to the method adopted by the European Union [PN-EN 13041: 2002]. Designations were made in the cylinders with a diameter of 5 cm and a height of 5 cm. The water-air capacity was determined with the 'Eijkelkamp' sand apparatus at a vacuum corresponding to a negative pressure of –3.2 cm H_2O. Water holding capacity, bulk density and porosity were calculated in accordance with PN-EN 13041 [2002]. The organic matter content was determined after sample incinerating in accordance with PN-EN 13039 [2002].

Statistical analysis

Data concerning statistical differences between intensively cultivated and fallow soil were tested by means of the Student two tailed test separately for each crop. If the homogeneity criterion of the variance tested by the Lavene'a method was not met, a modified generalisation of the test, the so-called Welch test, was used. The calculation were done using Statistica package v. 13.1 (Dell Inc. 2016).

RESULTS

Microorganisms and microbial activity in soils

Analyses of microbial groups in soil samples showed small differences between studied microorganisms, determined in intensively cultivated and fallow soils. The greatest differences were found in the case of onion cultivation, where the total number of fungi, bacteria and actinomycetes were significantly lower in the soil samples from under cultivation than in the fallow soils (Tab. 1). For other groups of studied microorganisms there were no differences between arable and fallow soils. In contrast, many years of intensive pepper cultivation had no effect on the total abundance of culturable microbial groups compared to the neighboring uncultivated soils. However, it was observed that "pepper's" soils indicated tendency to reduced number of overall bacteria, and especially fluorescent *Pseudomonas*, compared to soils from onion cultivation.

The studies of dehydrogenases showed a reduction in the activity of microorganisms in the soils with onion and pepper crops compared to the adjacent fallow land (Fig. 1). The highest difference was observed for onion cultivation, where DHA was determined on average at the level of 17.74 and 89.89 μg TFP g^{-1} of soil dry weight for intensive cultivated soil and fallow respectively. In the case of both types of the soils sampled in pepper production, the differences were not significant (34.81 and 46.29 μg TFP g^{-1}). Howev-

Table 1. The number of microorganisms in soils used for intensive vegetable cropping (intensive) and not cultured soils (fallow) presented as $\log_{10}$ cfu $g^{-1} \pm$ SE

Microorganisms	Onion		Pepper	
	intensive	fallow	intensive	fallow
Total fungi	5.1 ±0.3 b	5.3 ±0.4 a	5.3 ±0.3 a	5.4 ±0.4 a
Bacteria	7.4 ±0.4 b	7.6 ±0.3 a	7.2 ±0.4 a	7.2 ±0.2 a
Actinomycetes	6.6 ±0.4 b	6.9 ±0.3 a	6.6 ±0.3 a	6.7 ±0.3 a
Copiotrophs	10.1 ±0.5 a	10.0 ±0.5 a	10.4 ±0.7 a	10.3 ±0.5 a
Oligotrophs	9.3 ±0.9 a	9.1 ±0.9 a	9.3 ±0.7 a	9.4 ±0.6 a
Spore-forming bacteria	6.4 ±0.4 a	6.4 ±0.3 a	6.1 ±0.5 a	6.2 ±0.5 a
Fluorescent *Pseudomonas*	4.2 ±0.4 a	4.4 ±0.3 a	3.8 ±1.2 a	3.8 ±0.9 a

Means for cropping and fallow soils indicated by the same letter do not differ significantly according to Student test at p = 0.05

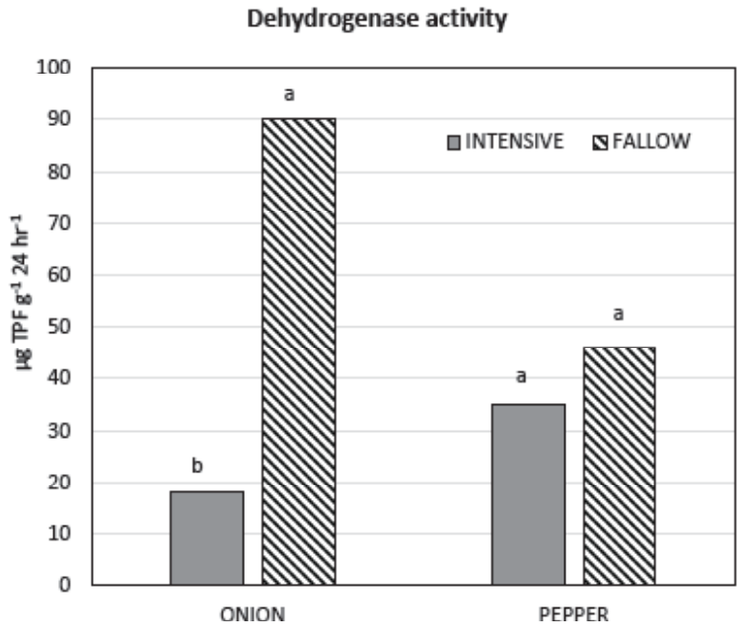

Fig. 1. Enzyme dehydrogenase activity in soils cropped with onion or pepper compared to the fallow land adjacent to these crops. Different letters above the bars indicated significant differences between means according to Welch test at p = 0.05, separately for each crop

er, the tendency to increased dehydrogenase activity in not cultivated soil, taken in the neighbourhood of growing tunnels, was observed. It was also noticed, that enzymes activity in pepper producing farms was markedly lower than in not cultivated soils adjacent to the fields of onion.

Chemical parameters of the soils

Soil management had no significant effect on pH (Tab. 2). However, there were differences between fields of onion and soils sampled in pepper production, which had distinctly lower pH (pH 6.4). In general, chemical parameters of soils from this type of produce (in tunnels) were different comparing to field cultivation of onion. The soil sampled in pepper tunnels characterized high salinity (EC 0.75 mS cm^{-1}), i.e. significantly higher than in control fallows and in the other examined soils (EC range 0.29–0.47 mS cm^{-1}). Similar effect was observed for nitrogen and phosphorus contents. Concentrations of $N\text{-}NO_3$ was in average 99.7 mg dm^{-3} of soil, while for onion this was 75.0 mg dm^{-3}. In the case of P the values were: 212.6 and 122.7 mg dm^{-3} for pepper and onion cultivated soils respectively. The opposite effect was observed for calcium whose contents were lower in pepper crops (1419 mg dm^{-3}) than in field crops of onion (1813 mg dm^{-3}).

Comparing nutrients concentrations between arable soils and fallows in studied crops, the most significant differences were again in the case of pepper followed by onion. The soils, where pepper was cultivated, contained significantly more $N\text{-}NO_3$, P, Ca, Cu and Zn than adjacent fallows, but less Fe. In onion cultivation salinity and nitrogen content were significantly higher, but K and Mg were lower than in not cultured soils. According to micronutrients the differences between cultivated soils and fallows were not significant.

Physical properties of the soils

The differences in the physical properties between arable soil and fallow were established for the cultivation of onions (Tab. 3). Cultured soil characterized significantly higher bulk density 1.4 g cm^{-3} and lower soil

porosity about 46%, compared to fallow, where soil bulk density was 1.1 g cm^{-3} and soil porosity ranged 55–56%. In the pepper producing farms there were no significant differences between cropped soil and soils in the vicinity of the tunnels. It was observed, that soil in the tunnels was less compact than in field vegetable production. Whereas fallow soil, taken in the vicinity of the tunnels, was more compacted – opposite to other fallows. In the case of water holding capacity, there were not significant differences between both types of soil use.

It was found that fallow soils characterized significantly higher organic matter content (SOM ranged 3.78–3.80%) than in soils used for vegetable cultivation (SOM ranged 2.24–2.82%) – as in Figure 2. The higher differences were determined for onion. In the case of pepper, the difference in SOM content between not cultivated soil and "tunnel" soil was not that much bigger, but still relevant.

Fertilization practices and crop rotation

On the basis of the surveys carried out in the visited farms, it was found that in field production of onion mineral fertilization prevailed (Fig. 3). In opposite, in pepper production combined mineral-organic fertilization was mainly used (in 60% of monitored farms). In the case of organic fertilizers cattle manure was the

Table 2. Concentration of nutrients (means in mg dm^{-3} of soil ± SE) in soils under intensive vegetable production and in fallow soils

Nutrients	Onion		Pepper	
	intensive	fallow	intensive	fallow
pH	6.8 ±1.2 a	7.2 ±1.0 a	6.4 ±0.7 a	6.3 ±0.8 a
EC (mS cm^{-1})	0.47 ±0.24 a	0.36 ±0.24 b	0.75 ±0.43 a	0.29 ±0.22 b
$N\text{-}NO_3$	75.0 ±76.3 a	40.4 ±32.4 b	99.7 ±87.0 a	29.6 ±15.4 b
P	122.7 ±75.1 a	116.6 ±65.6 a	212.6 ±115.5 a	73.4 ±50.9 b
K	139.8 ±74.8 b	184.9 ±123.4 a	120.6 ±82.6 a	119.8 ±75.8 a
Ca	1813 ±1467 a	2449 ±1731 a	1419 ±862 a	839 ±519 b
Mg	128.5 ±48.5 b	177.5 ±82.8 a	131.8 ±52.7 a	90.4 ±31.6 b
Fe	103.4 ±79.9 a	91.9 ±83.5 a	106.8 ±49.8 b	145.9 ±68.2 a
Mn	20.8 ±24.2 a	11.4 ±11.9 a	9.0 ±3.6 a	8.0 ±4.7 a
Cu	2.5 ±1.6 a	2.6 ±2.6 a	1.8 ±0.6 a	1.3 ±0.4 b
Zn	4.1 ±2.6 a	12.4 ±30.4 a	9.8 ±4.7 a	6.4 ±3.0 b
B	0.7 ±0.3 a	0.9 ±0.6 a	1.0 ±0.6 a	0.8 ±0.4 a

Means for cropping and fallow soils indicated by the same letter do not differ significantly according to Student or Welch test at p = 0.05

Table 3. Physical properties of studied soils (means ± SE)

Parameter	Onion		Pepper	
	intensive	fallow	intensive	fallow
Soil bulk density (g cm^{-3})	1.41 ±0.15 a	1.13 ±0.20 b	1.33 ±0.10 a	1.25 ±0.19 a
Soil porosity (%)	45.6 ±5.8 b	56.0 ±7.9 a	48.7 ±3.9 a	51.0 ±7.1 a
Water holding capacity (%)	41.6 ±4.2 a	44.3 ±8.2 a	45.7 ±2.9 a	46.6 ±3.6 a

Means for cropping and fallow soils indicated by the same letter do not differ significantly according to Student test at p = 0.05

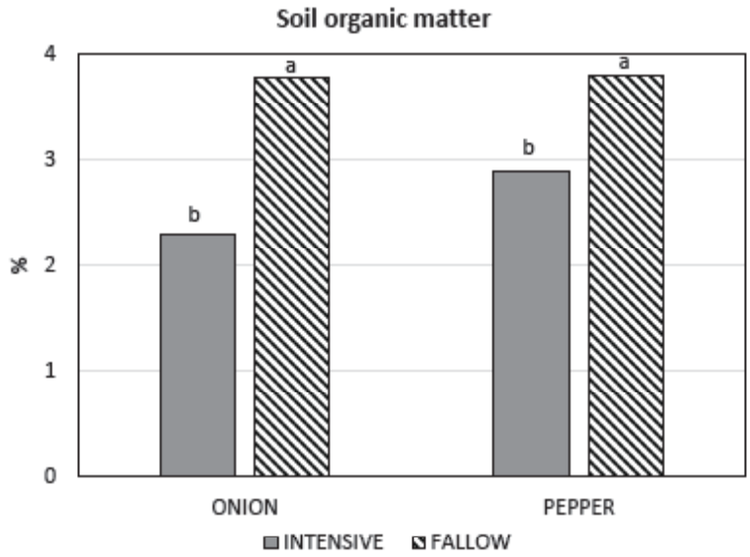

Fig. 2. Organic matter content (% vol.) in soils cropped with onion, cabbage or pepper compared to the fallow land adjacent to these crops. Different letters above bars indicated significant differences between means according to Student test at p = 0.05, separately for each crop

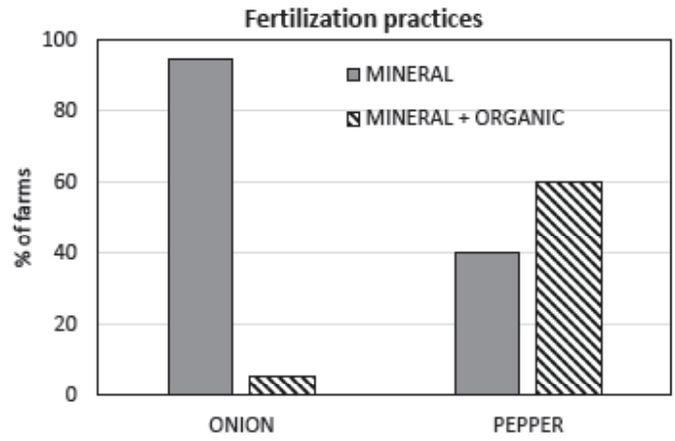

Fig. 3. Comparison of the rate of mineral and mineral-organic fertilization in production of onion and pepper

most frequently used. However, in pepper cultivation chicken dung applied in autumn, after harvest was also applied.

Analysis of the farmers' data has shown that rotation studied crops was very simplified. Onion in most cases was grown in monoculture or in rotation with potato and carrot, and occasionally with celery, lettuce or wheat. The most simplified rotation was in the cultivation of pepper. In eight out of ten visited farms, the crop was grown in a monoculture that lasts for several years or more (in one case it was 22 years). In one farm pepper was rotated with cucurbits and bean, and in second with barley. In two sites pepper was intercropped with radish or dill and lupine as green manure.

DISCUSSION

The investigations performed in this work show the effects of intensive onion and pepper cultivation on soil's biotic and abiotic characters, and indicate which of studied parameters are the most sensitive to practices employed in both crops production. The research showed significant functional degradation of cultivated soils expressed by reduced enzymatic microbial activity and organic matter content. Soil physical properties deteriorated the most in the field cultivation of onions. These factors may be considered as reliable indicators of soil health in vegetable production. The other studied parameters such as pH, nutrients availability and microbial abundance seems to be less sensitive. However, the evaluation of their values supported the hypotheses about the reasons for soil degradation in each of the crops studied.

Although soil quality deteriorated in both crop types, the causes were different. The most adverse soil conditions were found in onion cultivation. The studies have shown, that commonly applied agrotechnical system for this vegetable production has a strong negative impact on the biological status of the soils, as indicated by the low activity of dehydrogenases DHA and decreased organic matter contents in contrast to fallows. Moreover, these soils characterized the highest bulk density and the lowest porosity, and water holding capacity compared to non-cultivated soils, but also to the soil in the tunnels with pepper.

The activity of dehydrogenases, as a group of enzymes acting intracellularly, informs about the biologically active microbial population in soil [Wolińska and Stępniewska 2012, Błońska et al. 2017]. Therefore, measurements of the activity of these enzymes are used to assess soil quality and the impact of soil use and degradation/regeneration rates, and their higher activity indicates higher functional diversity and soil fertility [Gil-Sotres 2005, Raiesi and Behesthi 2015]. There are several factors that may have impact on soil dehydrogenases activity. Among them the most important are SOM content, soil moisture, soil aeration [Wolińska et al. 2015].

The amount of organic matter is one of the most important indicators of soil fertility [Scotti et al. 2015, Bashir et al. 2023]. A high content of OM is a factor stabilising the structure, reducing the susceptibility of soil to compaction, erosion and degradation. The relationship between DHA and SOM and worsened soil physical soil properties are also clearly visible in our research. The highest DHA and SOM content were in the soil collected form fallows. These values strongly decreased in cultured soils. Organic matter enhances soil physical and chemical properties and promotes biological activity [Chakraborty et al. 2011], affecting the supply of energy for microbial growth and enzyme production [Fontaine et al. 2003]. As dehydrogenase activity reflects metabolic activity of the soil [Mohammadi et al. 2011], it may be considered to be proportional to the biomass of the microorganisms [Wolińska et al. 2015]. In our studies, in onion production, the number of fungi bacteria and actinomycetes in cultivated soils was significantly lower than in fallows. Similarly, Bonanomi et al. [2011] compared the biological characteristics of soils collected from different agricultural farms and found a drastic reduction of soil microbial biomass and enzymatic activities in soils having small organic C content and under intensive agriculture, without use of organic amendments. In turn Lu et al. [2021] concluded that excessive application of mineral fertilizers in vegetable production has led to the substantial soil enrichment of P, K, Ca and Mg and soil acidification at 0–60 cm depths, and resulted in rapid decrease of SOC content, followed by decline in soil physical and biological properties. In our case over-fertilisation was not observed in onion cultivation. The concentrations of nutrients in cultivated soils and in fallows were comparable, and soil acidification did not occur. It suggests, that the farmers fertilized the crops according to plant requirements, recommended by the local extension services. However, it was reported that low humus reserves in Polish soils are also related to its intensive mineralization, removal of organic residues, while limiting the supply of organic material to the soil [Jończyk et al. 2008, Smreczak and Jadczyszyn 2017]. Onion is one of such crops, where organic residues are minor and mostly transferred from the field. Moreover, the soil in onion production for the most time is not covered with vegetation. Weeds are intensively controlled with herbicides, because onion is very sensitive for competition with other plants. Intensive control of weeds, pests and pathogens, split-dose fertilisers application [Jarecka-Boncela et al. 2017] and growing

need for irrigation require numerous passes by heavy equipment. This led to soil compaction, its structure degradation, and in consequence to increase organic matter mineralization and lower carbon sequestration [Jakab et al. 2023]. This is repeated year after year, especially that such production is often practised in monoculture.

In pepper cultivation, despite of less "invasive" tillage (the use of heavy equipment is not possible under tunnels) and more frequent application of organic manures (Fig. 3), the situation was not much better compared to onion production. Soil compaction in tunnels was less severe, but SOM content was comparable to soils from onion production. Moreover, excessive application of mineral fertilizers caused high concentration of macro-elements resulting in high salinity and acidification of the soil. Conditions in tunnels are favorable for pests and diseases. Therefore, intensive pepper monoculture leads to an accumulation of pathogens and pests, and the resulting increased use of pesticides. Microbial communities are also strongly affected by fumigants and pesticides usage [Jacobsen and Hjelmsø 2014]. Ciarkowska and Sołek-Podwika [2012] reported that long-term growing of vegetables in monoculture, in foil tunnels reduces urease activity in soil reflecting the changes in soil environment, and have pointed out the hampering effect of frequently used pesticides. Intensive mineral fertilization and pesticide management are also listed as a factors inhibiting dehydrogenase activity [Chang et al. 2007, Luo et al. 2015], what was observed in our studies.

Mar Guerrero et al. [2014] have found, that soil fatigue caused by continuous cropping of pepper plants at the same site is highly specific towards this crop, and does not apply to other plant species cultivated in this soil. Whereas, according to Liang et al. [2013] intensive re-cropping of the same vegetables may negatively affect soil organisms communities changing their metabolism. They showed that repetitive cropping of cucumbers enhanced population of fungi in the soil. In presented studies, the number of fungi in pepper tunnels did not differ compared to other soil samples. However, markedly lower abundance of bacteria was observed. Thus, the proportions of fungi in the microbial community increased in these soils. This could be effect of long-term, repeated mineral fertilizers application, especially nitrogen inputs, which lead to acidification and suppress soil microorganisms [Tian et al. 2015], causing adverse changes in microbial communities, favoring fungi and increasing fungal biomass in relation to bacterial biomass. The soil from pepper production contained also the lowest abundance of *Pseudomonas* bacteria, recognised as beneficial to plants [Mehmood et al. 2023]. These results suggest that, although, generally no significant changes in total microbial abundance were observed in studied soils, the adverse alterations may have occurred in microbiota composition and biodiversity. However, it needs more detailed studies with the use of culture-independent molecular techniques such as next generation sequencing (NGS) to characterize the differences in soil microbiome's composition.

CONCLUSIONS

The results showed that intensive cultivation of economically important vegetables grown in Poland such as onion and pepper, has detrimental effect on soil environment. Despite of each crop management affect the soil *via* different factors, the most significant adverse effects were reduction of DHA and SOM content. Both of them may be considered as reliable indicators of soil health condition in vegetable production. However, in order to understand the causes of the phenomena, other soil parameters also need to be thoroughly investigated.

In onion production, multiple operations with the use of agricultural equipment lead to a breakdown of soil structure. This effect is exacerbated by the poor surface coverage with vegetation related to intensive weeds control with herbicides, and minor supply of organic material to soil. Despite the use of rational mineral fertilization, these factors led to soil compaction, reduced water infiltration, surface erosion, decrease of soil organic matter, suppression of the biological functioning, and in consequence degradation of the soil. The situation is worsened by onion re-cropping for several years. Therefore reduction of tillage practices, intercrops implementation and regular crop rotation should be recommended in onion production.

In turn, in pepper cultivation, frequent cropping of the same plant for years on the same site, chemical soil disinfection frequently applied against soil-borne pathogens, with high mineral fertilizer application

rates causes soil acidification and adverse effect on soil microorganisms, increasing the proportion of fungi in microbial community. Even substantial application of organic manuring in pepper monoculture did not reduce this detrimental effect. High mineral input in pepper production may cause an increase of soil organic matter mineralization. Therefore, more precise and reasonable application of fertilizers and disinfectants should be recommended. One of the solution could be also to break pepper monoculture by rotation with other crops, which can be cultivated under cover. The results indicate that future sustainable vegetable cropping systems should include a more efficient crop rotation, with fewer tillage operations and the implementation of practices leading to an increase in organic matter resources

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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