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RESEARCH PAPER

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EFFECTS OF NPK FERTILIZATION, CULTIVAR, AND ROW SPACING ON SEED YIELD AND FATTY ACID COMPOSITION OF AMARANTH (*Amaranthus* spp.)

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ABSTRACT

Amaranth (*Amaranthus* spp.) is a pseudo-cereal with increasing agronomic and nutritional importance due to its high seed yield potential and valuable lipid composition. The aim of this study was to evaluate the effects of NPK fertilization level, cultivar, row spacing and their interactions on yield components, crude fat content, fat yield and fatty acid composition of amaranth seeds. A three-year field experiment (2016–2018) was conducted in southeastern Poland using two cultivars (Rawa and Aztek), four levels of NPK fertilization and two row spacings (30 and 55 cm). The results showed that cultivar was the main factor differentiating seed yield, crude fat content, fat yield and fatty acid composition. The Aztek cultivar produced significantly higher seed yield and fat yield, whereas Rawa was characterized by a higher proportion of unsaturated fatty acids. Row spacing significantly affected plant density and yield structure, while increasing NPK fertilization enhanced seed and fat yield. Significant two-factor interactions were identified. The interaction between fertilization level and cultivar significantly affected palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids, whereas the interaction between row spacing and cultivar influenced linoleic acid content ($C_{18:2}$, n-6) and the n-6/n-3 fatty acid ratio. These results confirm that both quantitative and qualitative traits of amaranth seeds are determined by complex interactions between genetic and agrotechnical factors.

Keywords: amaranth, NPK fertilization, cultivar, row spacing, yield structure, fat content

INTRODUCTION

Amaranth (*Amaranthus* spp.) is a multifunctional crop characterized by high aboveground biomass production, considerable plant height, and well-developed inflorescences, which makes it attractive for a wide range of agricultural, horticultural, and environmental applications, including animal feeding, ornamental use, and soil protection [Schafleitner et al. 2022, Jan et al. 2023]. Field and controlled-environment studies have demonstrated that the growth performance and biomass production of amaranth are strongly influenced by cultivation system and environmental conditions, with substantial differences observed between open-field and protected cultivation; in particular, Managa et al. [2023] reported pronounced effects of production system on growth dynamics and mineral composition of amaranth biomass, highlighting the crop's high phenotypic plasticity.

In recent years, increasing attention has been paid to amaranth seeds, which are valued for their high nutritional quality, particularly their protein content, oil fraction, and abundance of bioactive compounds such as

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squalene, tocopherols, and phytosterols [Skwaryło-Bednarz et al. 2020, Jan et al. 2023, Malik et al. 2023]. Among amaranth species, *Amaranthus cruentus*, *A. hypochondriacus*, and *A. caudatus* are recognized as species with the highest nutritional value of seeds and considerable agronomic potential under diverse environmental conditions [El Gendy et al. 2018, Skwaryło-Bednarz et al. 2020]. Amaranth seed oil typically constitutes approximately 5–10% (with a mean value of about 7.0%) of seed dry matter and is characterized by a favorable fatty acid composition a dominated by unsaturated fatty acids, mainly linoleic acid $C_{18:2}$ (n-6) and oleic acid $C_{18:1}$ (n-9), which together represent the predominant fraction of seed lipids [Baraniak and Kania-Dobrowolska 2022, Azri et al. 2025].

Beyond its fatty acid profile, amaranth seed oil is distinguished by a high content of unsaponifiable bioactive compounds, particularly squalene, tocopherols, and phytosterols, which contribute to its antioxidant properties and support its utilization in food, cosmetic, and pharmaceutical applications, underlining the multifunctional nature of this crop [Sayed-Ahmad et al. 2022]. Beyond total oil content, increasing attention has been paid to the qualitative composition of fatty acids, especially the ratio of omega-6 (n-6) to omega-3 (n-3) fatty acids, which is considered a key indicator of nutritional quality and health-promoting potential of plant oils. An excessive n-6/n-3 ratio has been associated with metabolic and cardiovascular disorders, whereas lower values are regarded as more desirable from a dietary perspective [Simopoulos 2016, Calder 2017]. Although amaranth oil is rich in bioactive compounds with documented antioxidant and hypolipidemic properties, its n-6/n-3 ratio is generally high, often exceeding 30:1, which may limit its suitability as a primary dietary oil [USDA FoodData Central 2023].

Recent studies indicate that the fatty acid composition of amaranth oil is influenced not only by genetic factors but also by environmental conditions and agronomic practices, including nutrient availability and cultivation intensity [Tyrus et al. 2024]. Nevertheless, despite growing interest in amaranth as a functional crop, there is still limited information on how genotype interacts with agronomic factors such as fertilization level and row spacing to shape fatty acid profiles and their ratios, particularly under temperate European climatic conditions. This research gap is especially relevant in the context of sustainable nutrient management and breeding strategies that increasingly focus on improving oil quality and nutritional value rather than yield alone [Petkova et al. 2019, Jan et al. 2023, Azri et al. 2025].

Cultivars representing different *Amaranthus* species may differ in their physiological response to nutrient supply, lipid biosynthesis pathways, and stress tolerance, which may ultimately affect both the quantity and quality of seed oil [Schafleitner et al. 2022, Azri et al. 2025]. Recent comprehensive reviews have emphasized the high nutritional and nutraceutical potential of amaranth seeds, particularly in relation to their lipid fraction rich in unsaturated fatty acids, tocopherols, and other bioactive compounds, while simultaneously highlighting the scarcity of field-based experiments linking agronomic practices with fatty acid composition and quality parameters under realistic cultivation conditions [Jan et al. 2023].

Improving the nutritional quality of amaranth seed oil, rather than increasing oil yield alone, is increasingly considered a key objective in sustainable crop production and functional food development. In this context, fatty acid composition and their relative proportions are regarded as critical quality indicators determining the health-promoting potential of amaranth-derived products [Jan et al. 2023].

Therefore, the objective of this study was to evaluate the effects of differentiated NPK fertilization, row spacing, and cultivar on selected morphological traits, seed yield, oil content, and fatty acid composition of two amaranth cultivars: Rawa (*Amaranthus cruentus* L.) and Aztek (*Amaranthus hypochondriacus* × *A. hybridus* L.), cultivated under the soil and climatic conditions of southeastern Poland. The null hypothesis (H_0) assumes that NPK fertilization and row spacing would not significantly affect morphological traits, seed yield, oil content, or the fatty acid composition of the studied cultivars. The alternative hypothesis (H_1) assumes that increased fertilization intensity and wider row spacing would significantly influence these traits and that the magnitude of these effects would depend on cultivar-specific responses.

MATERIALS AND METHODS

Field experiment description

This study was based on a field experiment conducted from 2016 to 2018 on a private farm field located in the village of Bodaczów (50°71'N, 23°04'E), near Zamość, in the Lublin Voivodeship. The experiment was established using a split-plot design arranged in randomized blocks with three replications. The experimental factors included NPK fertilization, cultivar, and row spacing. Each elementary plot covered an area of 10 m² (2 m × 5 m). Within each replication (block), plots were arranged in a compact layout with 0.5 m wide alleys between adjacent plots and

1.0 m wide alleys between blocks to allow for machinery access and to minimize edge effects. The total experimental area, including plots and alleys, was approximately 720 m^2 per year. The experiment was set up on brown soil developed from loess. Each year, prior to establishing the experiment, soil samples were collected for chemical analysis to determine the levels of phosphorus (P), potassium (K), magnesium (Mg), and soil pH. The soil's nutrient content in terms of P, K, and Mg was classified as high, while the soil pH was neutral.

The preceding crops for amaranth were: spring barley in 2016, spring wheat with white mustard as an intercrop in 2017, and winter wheat in 2018.

The experiment included the following variables: NPK fertilization (n = 4), cultivars (n = 2), row spacing (n = 2), and replications (n = 3), for a total of 48 plots:

I. NPK fertilization levels (kg·ha^{-1}):

1. NPK0 (Control) – 0 N, 0 P_2O_5, 0 K_2O
2. NPK1 – 80 N, 50 P_2O_5, 50 K_2O (equivalent to 80 N, 22 P, 41.5 K = 143.5 NPK)
3. NPK2 – 110 N, 70 P_2O_5, 70 K_2O (110 N, 30.8 P, 58.1 K = 198.9 NPK)
4. NPK3 – 140 N, 90 P_2O_5, 90 K_2O (140 N, 39.6 P, 74.7 K = 254.3 NPK)

II. Cultivars:

1. Rawa (*A. cruentus* L.)
2. Aztek (*A. hypochondriacus* × *A. hybridus* L.)

III. Row spacing:

1. 30 cm
2. 55 cm

All tillage and cultivation practices were carried out in accordance with standard agronomic recommendations for amaranth. Seeds of two amaranth cultivars (Rawa and Aztek) with certified quality were sown at a rate of 2.0 kg·ha^{-1} using a plot seeder (Tool Carrier 2700). In narrow-row sowing (30 cm), plants were sown in four rows per 1 m^2, with the first row located approximately 25 cm from the longer edge of the plot. In wide-row sowing (55 cm), rows were spaced evenly, with the first row located approximately 45 cm from the longer edge of the plot. Border rows and border plants were excluded from sampling and measurements; all observations and harvests were performed only from the central area of each plot to eliminate edge effects. Sowing was performed in the third decade of May (2016 and 2017) or the first decade of June (2018). In each year of the experiment, certified seed material was provided free of charge by "Szarłat" M. and W. Lenkiewicz sp.j.

Phosphorus (P) fertilization in the form of granular single superphosphate and potassium (K) fertilization were applied in autumn. Nitrogen (N), in the form of ammonium nitrate, was applied to the soil in spring in two equal split doses: the first before sowing and the second four weeks after plant emergence.

Each season, the experimental field underwent two rounds of weed control, combining manual and mechanical methods. No chemical protection against agrophages (pathogens and pests) was applied, due to the lack of registered plant protection products for amaranth; therefore, crop protection relied solely on mechanical and manual methods. Harvesting of seeds was carried out manually in the third decade of October (2016) and the first decade of November (2017 and 2018). Post-harvest, seed moisture content was high and required rapid drying to maintain quality and health standards. Moisture content directly after threshing was 28.9% in 2016, 21.3% in 2017, and 24.4% in 2018. Seeds were dried each year to approximately 10.0% moisture.

Weather conditions

Meteorological data for the study period (2016–2018) were obtained from the Institute of Meteorology and Water Management – National Research Institute (IMGW) at the nearest weather station in Zamość. The following parameters were analyzed: average monthly air temperature (°C) and total monthly precipitation (mm) during the amaranth growing season (May to October).

Assessment of plant density per 1 m^2 and selected morphological traits before harvest

At the BBCH 89 growth stage, the number of plants per 1 m^2 (plants/m^2) was counted prior to harvest, and biometric measurements were performed on 30 randomly selected plants from each plot. The following traits were evaluated: plant height (cm), inflorescence length (cm), seed mass per plant (g), and thousand seed weight (g) [Martínez-Núñez et al. 2019].

After seed harvest at the BBCH 92 stage, seed yield was determined in kg per plot and converted to t·ha^{-1}. From each plot, a 0.5 kg seed sample was collected for further analyses of chemical composition.

Chemical analyses

Each year, the following chemical analyses were conducted on the harvested seeds of cultivated amaranth:

- Crude fat content was determined according to the Polish Standard PN-EN ISO 6492:2005 Animal feeding stuffs – Determination of fat content [PN-79R-65950];
- Fatty acid composition was determined by gas chromatography. The analysis was carried out using a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionization detector (FID). Prior to analysis, triglycerides were transesterified into fatty acid methyl esters (FAMEs) using potassium methanolate. Fatty acid separation was performed on an Rtx-2330 capillary column (105 m × 0.32 mm, 0.20 μm).

Statistical analysis

The results are presented as averages for each year of the study. The data were analyzed using analysis of variance (ANOVA) appropriate for a split-plot design in a randomized block arrangement. The experimental factors included NPK fertilization, cultivar, and row spacing, as well as their interactions. All two-factor interactions between experimental factors were included in the statistical model and evaluated accordingly. The null hypothesis (H_0) was tested using Snedecor's F-test, and the corresponding probabilities were determined. Year was included in the analysis as an environmental factor, and treatment effects were evaluated across years. Differences between means were assessed using Tukey's honestly significant difference (HSD) test at a significance level of $\alpha = 0.05$. To assess data variability, the coefficient of variation (CV%) was calculated. Relationships between selected traits were examined using Pearson correlation and linear regression analyses. All statistical analyses were performed using Excel 7.0 and Statistica software (StatSoft Polska, 2013).

RESULTS AND DISCUSSION

Environmental conditions during the study period

Weather conditions during the study years were highly variable (Figure 1).

In 2016, amaranth was found to be sown during a wet and generally warm May, with total precipitation amounting to 49.9 mm and an average monthly temperature of 14.3°C (Figure 1). Throughout the growing season, mean air temperatures consistently exceeded the long-term average. Precipitation levels from June to October also remained above the climatological norm. Notably, October recorded an exceptionally high total precipitation of 99.9 mm, more than double the long-term average, which considerably hindered the seed harvest.

In 2017, sowing occurred in a warm spring characterized by a marked precipitation deficit (52.2 mm) and an average temperature of 13.7°C (Figure 1). Despite temperatures generally remaining above the long-term mean, they were lower than in 2016, except in July and August. Cumulative rainfall throughout the growing season was substantially below average, adversely affecting seed germination, plant development, and biomass accumulation. September (67.7 mm) and October (66.0 mm) were notably wet, significantly delaying seed maturation and complicating harvest operations.

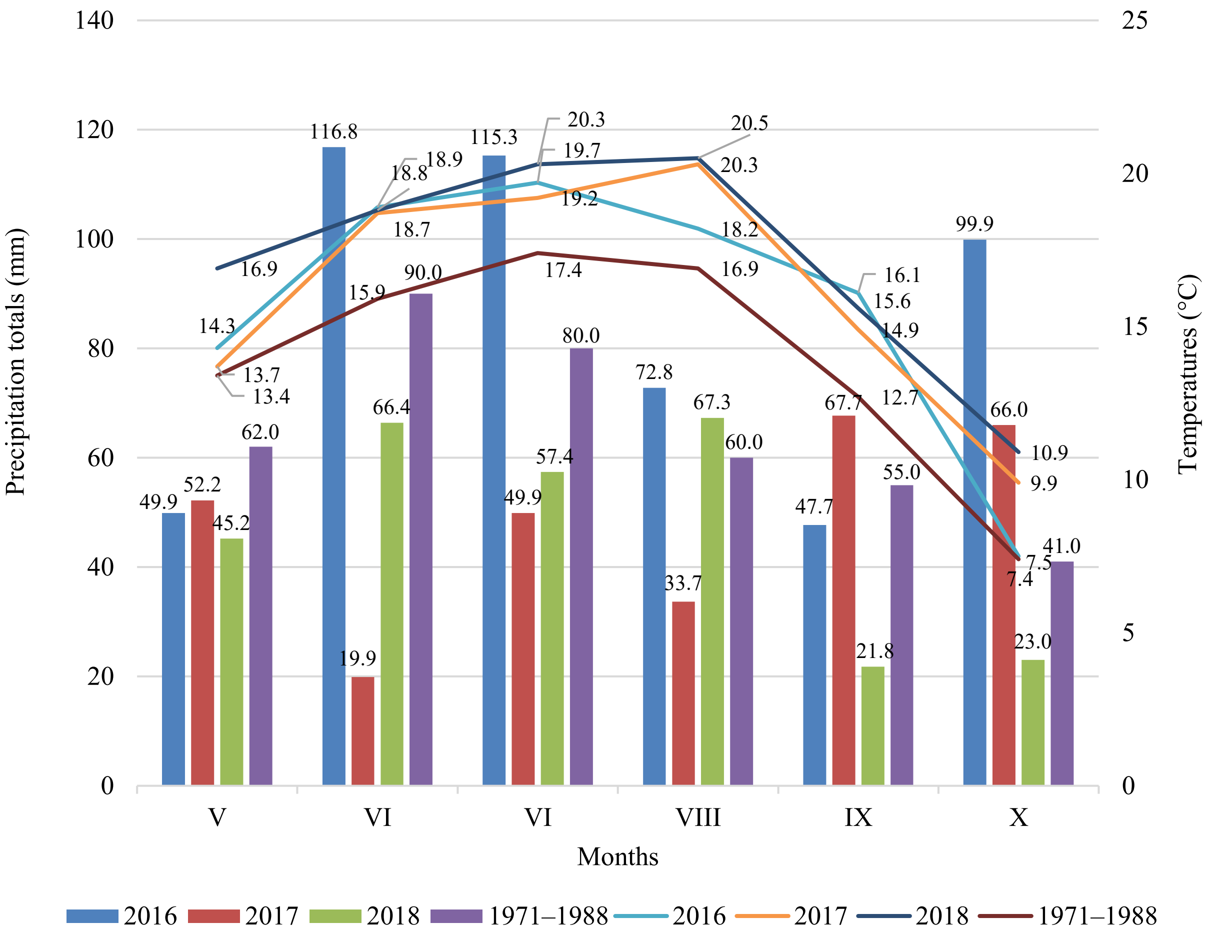
In 2018, the entire growing season – from sowing to harvest – was marked by temperatures consistently surpassing long-term norms, making it the warmest year of the study (Figure 1). May experienced the most severe rainfall deficit of the experimental period (45.2 mm; 37.2% below the long-term average). Although July remained relatively dry, precipitation levels were higher than those observed in 2017. August precipitation slightly exceeded the long-term average by 7.3 mm. Favorably low precipitation in October facilitated efficient seed harvest.

Plant stand structure and morphological traits

A synthesis of the three-year research results demonstrated that plant density was a key factor shaping yield structure in amaranth cultivation. In the present study, the number of plants per square meter was significantly affected only by row spacing, either 30 cm (narrow-row) or 55 cm (wide-row) – as in Table 1. This trait showed relatively high variability, as reflected by the average coefficient of variation of 26.82% (Table 1), indicating a strong dependence on stand structure rather than genetic control.

Proper plant density is a critical determinant of uniform plant height and appropriate development within the canopy. For amaranth, it has been suggested that maintaining a plant population per square meter that allows optimal inflorescence development is particularly important, as inflorescences may account for approximately 50% of the total plant biomass [Kozak et al. 2011]. However, achieving such stand density can be challenging under field conditions, especially for the Rawa cultivar, due to unfavorable weather during establishment and early growth stages [Jendrzejczak and Śmigerska 2014].

Figure 1. Average monthly air temperatures (°C) and precipitation totals (mm) for the years 2016–2018 and the long-term averages for 1971–1988 (Meteorological Station in Zamość)



Excessive plant density per unit area is known to increase the proportion of vegetative organs, such as leaves and stems, and to promote plant lodging and stem breakage under conditions of strong wind and heavy rainfall [Skwaryło-Bednarz and Nalborczyk 2006]. Dense stands may also enhance susceptibility to fungal diseases [Pusz et al. 2015]. The results showed that narrow-row sowing with dense plant growth resulted mainly in the development of a single main stem bearing one inflorescence, whereas wider spacing favored stem branching and the formation of multiple inflorescences. Conversely, lower plant density per unit area may positively affect seed quality traits, including fat content, as previously reported for amaranth [Kozak et al. 2011].

In the present study, plant height exhibited very low variability (CV = 5.80), while inflorescence length showed moderate variability (CV = 25.25) – as in Table 1. Both morphological traits were significantly influenced only by cultivar. Averaged across the three growing seasons, the Aztek cultivar was taller by 16.9 cm and produced inflorescences that were 19.5 cm longer than those of Rawa (Table 1). These results are consistent with earlier findings indicating substantial cultivar-dependent differences in amaranth plant architecture [Kozak et al. 2011, Schafleitner et al. 2022].

Seed weight per plant was significantly affected only by row spacing (Table 1). Wide-row sowing (55 cm) enhanced growth dynamics by promoting stem branching and the formation of additional inflorescences, resulting in a higher number of seeds compared with narrow-row sowing (30 cm), with an average difference of 11.3 g per plant. The coefficient of variation for seed weight per plant reached 31.09%, indicating moderate variability of this trait (Table 1). Similar positive effects of reduced plant density and wider spacing on amaranth seed production have been reported under various environmental conditions [Olofintoye et al. 2015, Akamine et al. 2021, Tyrus et al. 2024]. Under favorable growing conditions, including low plant density, amaranth is capable of exceptionally high reproductive output. Gontarczyk [1996] reported that a single plant may produce more than 500 g of seeds.

In the present study, the 1000-seed weight was primarily influenced by hydrothermal conditions prevailing during the study years, as well as by cultivar and row spacing. In contrast, no significant relationships were observed between the analyzed morphological traits and increasing NPK fertilization levels (Table 1). This lack of response suggests that, under the conditions of the present experiment, fertilization intensity played a secondary role in shaping amaranth morphology. The very low coefficient of variation for 1000-seed weight (CV = 2.53%) indicates high stability of this trait across experimental treatments (Table 1). Nevertheless, previous studies have shown that the response of amaranth morphological traits to mineral fertilization may depend strongly on genotype, environmental conditions, and cultivation system [Akamine et al. 2021, Mndzebele et al. 2023, Tyrus et al. 2024]. Moreover, it has been emphasized that weather variability and stand structure frequently override the direct effects of fertilization on plant morphology, particularly under temperate and humid climatic conditions [Schafleitner et al. 2022, Azri et al. 2025].

Table 1. Number of plants per 1 m^2 and selected morphological traits before harvest (averages for factors and years)

Factor	Number of plants per 1 m^2 before harvest (pcs)	Plant height (cm)	Inflorescence length (cm)	Seed mass per plant (g)	Thousand seed weight (g)
NPK0	13.9 a	174.6 a	42.1 a	19.4 a	0.71 a
NPK1	14.0 a	178.3 a	45.7 a	20.0 a	0.72 a
NPK2	14.2 a	181.1 a	46.4 a	20.6 a	0.73 a
NPK3	14.2 a	183.1 a	46.8 a	21.5 a	0.71 a
$LSD_{0.05}$	4.3	11.3	12.8	7.2	0.03
F value	0.01	1.6	0.4	0.2	2.6
p	n.s.	n.s.	n.s.	n.s.	n.s.
A	14.1 a	187.7 a	55.0 a	21.9 a	0.71 b
R	14.0 a	170.8 b	35.5 b	18.9 a	0.73 a
$LSD_{0.05}$	2.2	3.6	3.6	3.7	0.01
F value	0.01	87.4	121.6	2.7	12.2
p	n.s.	***	***	n.s.	**
N	17.8 a	176.6 a	42.5 a	14.7 b	0.71 b
W	10.3 b	181.9 a	48.0 a	26.0 a	0.73 a
$LSD_{0.05}$	0.3	6.0	6.6	1.7	0.01
F value	2008.0	3.1	2.9	185.8	9.9
p	***	n.s.	n.s.	***	**
2016	14.6 a	184.1 a	47.6 a	22.3 a	0.73 a
2017	13.9 a	176.0 a	41.6 a	17.9 a	0.70 c
2018	13.9 a	177.6 a	46.5 a	20.9 a	0.72 b
$LSD_{0.05}$	3.3	8.7	9.8	5.4	0.01
F value	0.1	2.9	1.3	2.1	11.16
p	n.s.	n.s.	n.s.	n.s.	***
CV (%)	26.82	5.80	25.25	31.09	2.53

Legend: NPK0–NPK3 – fertilization levels; A – Aztek cultivar, R – Rawa cultivar; N – narrow row spacing, W – wide row spacing; 2016–2018 – study years; values followed by the same letters do not differ significantly at $p \leq 0.05$; *, **, and *** indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively; CV – coefficient of variation

The results of our study showed that only the interaction between weather conditions during the study years and row spacing significantly affected amaranth plant density per 1 m^2 before harvest (Table 2). No significant interaction effects were detected for the other factors, and no significant interactions among the studied factors were observed for the other analyzed morphological traits. These findings indicate that amaranth plant density is shaped predominantly by environmental variability and stand structure rather than by interactions among agronomic factors, which is consistent with reports highlighting the dominant role of climatic conditions and plant density in determining amaranth morphological traits [Akamine et al. 2021, Schafleitner et al. 2022, Mndzebele et al. 2023, Tyrus et al. 2024].

Table 2. Significant two-factor interactions affecting yield components and fatty acid composition of amaranth

Trait	Interaction	F value	Significance
Number of plants per 1 m^2 before harvest (pcs)	year × row spacing	3.90	*
Seed yield (t·ha $^{-1}$)	year × cultivar	4.95	*
Palmitic acid $C_{16:0}$ (%)	fertilization × cultivar	5.07	*
Stearic acid $C_{18:0}$ (%)	fertilization × cultivar	5.63	*
Linoleic acid $C_{18:2}$ (n-6) (%)	row spacing × cultivar	6.90	**
n-6/n-3 fatty acid ratio	row spacing × cultivar	7.46	**

Only statistically significant two-factor interactions are presented
*, ** indicate significance at $p \le 0.05$ and $p \le 0.01$, respectively

Seed yield and its determinants

The seed yield of cultivated amaranth was influenced to varying degrees by the studied factors, including fertilization level, row spacing, cultivar, and weather conditions, and was characterized by relatively low variability (CV = 12.33%) – as in Table 3. In the present study, seed yield depended primarily on hydrothermal conditions during the study years rather than on cultivar or the level of NPK fertilization, whereas row spacing (30 or 55 cm) did not significantly affect this trait (Table 3).

The significantly highest seed yield was recorded in the first year of the experiment (2016), exceeding the yield obtained in the second year (2017) by 22.0%, and that in the third year (2018) by 8.9% (Table 3). These differences can be attributed mainly to variable weather conditions in the Zamość region during the study years. In the first year, the precipitation sum (402.5 mm) recorded during the amaranth growing period (from June to early September) exceeding the long-term average, and positively affected seed yield despite sowing into poorly moistened soil. In contrast, in the second and third year of the study low precipitation (223.4 mm and 258.1 mm, respectively), their unfavorable distribution, and prolonged drought periods during vegetation limited yield formation and final yield size (Figure 1). Air temperature also influenced yield formation, as mean temperatures during all study years exceeded the long-term average, with the warmest conditions recorded in 2018, followed by 2016 and 2017.

The obtained results confirm earlier reports indicating that amaranth exhibits relatively high, although differentiated, tolerance to variable moisture conditions. This tolerance is partly related to its classification as a C_4 photosynthesis plant, characterized by high water-use efficiency and effective CO_2 fixation associated with efficient utilization of solar radiation [Gontarczyk 1996, Achremowicz et al. 2015, Schafleitner et al. 2022]. Nevertheless, amaranth is highly sensitive to soil water deficiency, particularly during the emergence and flowering stages [Olufolaji et al. 2010, Azri et al. 2025]. Insufficient soil moisture significantly delays emergence and contributes to plant thinning, while drought during inflorescence formation impairs flowering and fruit set, leading to reduced seed yield [Graham 2010, Grobelnik Mlakar et al. 2012]. Hydrothermal conditions during the growing period are therefore crucial not only for pseudo-cereal crops, which largely belong to the C_4 photosynthesis group, but also for cereal species predominantly classified as C_3 plants [Haliniarz 2013].

Cultivar was another important factor determining amaranth seed yield. Averaged across the study years, the Aztek cultivar produced a significantly higher seed yield than Rawa, with a difference of approximately 13% in favor of Aztek (Table 3). These results support previous findings indicating that genotype plays a decisive role in yield formation of amaranth under diverse environmental conditions. Studies conducted in Poland and other European regions, as well as in tropical and subtropical environments of East Africa (Tanzania) and East Asia (Taiwan), have consistently shown that cultivars related to *A. hypochondriacus* are characterized by higher yield potential compared with *A. cruentus* [Kozak et al. 2011, Skwaryło-Bednarz et al. 2011, Schafleitner et al. 2022, Tyrus et al. 2024]. According to these authors, the higher yield potential of the Aztek results not only from increased production of generative organs, but also from greater vegetative biomass accumulation, which may enhance assimilate supply to reproductive structures. The stronger yield response of Aztek to increased fertilization observed in this study may therefore be associated with higher growth vigor, more efficient nitrogen utilization, and greater allocation of assimilates to seed production, traits commonly reported for genotypes derived from *A. hypochondriacus*.

Table 3. Seed yield. crude fat content. and fat yield in amaranth seeds (averages for years and factors)

Factor	Seed yield ($t \cdot ha^{-1}$)	Crude fat content ($g \cdot kg^{-1}$)	Fat yield ($t \cdot ha^{-1}$)
NPK0	2.48 b	65.93 b	0.16 b
NPK1	2.57 ab	67.49 ab	0.17 ab
NPK2	2.71 ab	68.98 a	0.19 a
NPK3	2.82 a	70.00 a	0.20 a
$LSD_{0.05}$	0.03	2.65	0.03
F value	2.75	2.82	3.85
p	*	***	*
A	2.83 a	69.58 a	0.20 a
R	2.46 b	66.63 b	0.16 b
$LSD_{0.05}$	0.16	1.41	0.01
F value	22.92	17.85	23.09
p	***	***	***
N	2.61 a	67.69 a	0.18 a
W	2.68 a	68.51 a	0.18 a
$LSD_{0.05}$	0.19	1.64	0.02
F value	0.59	1.03	0.79
p	n.s.	n.s.	n.s.
2016	2.90 a	68.89 a	0.20 a
2017	2.38 c	65.99 b	0.16 c
2018	2.66 b	69.42 a	0.19 b
$LSD_{0.05}$	0.22	2.08	0.02
F value	16.95	9.24	13.96
p	***	***	***
CV (%)	12.33	4.10	15.85

Legend: NPK0–NPK3 – fertilization levels; A – Aztek cultivar, R – Rawa cultivar; N – narrow row spacing, W – wide row spacing; 2016–2018 – study years; values followed by the same letters do not differ significantly at $p \le 0.05$; *, **, and *** indicate significance at $p \le 0.05$, 0.01, and 0.001, respectively; CV – coefficient of variation

Compared with weather conditions and cultivar effects, NPK fertilization exerted a weaker but still significant influence on seed yield. Statistical analysis indicated that increasing macronutrient fertilization significantly increased seed yield, with the highest yield recorded at the third fertilization level (140 kg N, 90 kg P_2O_5, and 90 kg $K_2O \cdot ha^{-1}$), which was 13.6% higher than the unfertilized control. Numerous studies have emphasized the importance of mineral nutrition, particularly nitrogen, in shaping the yield of pseudo-cereal crops, including amaranth [Skwaryło-Bednarz 2010, Kozak et al. 2011, Bielski and Szwejkowska 2015, Akamine et al. 2021, Mndzebele et al. 2023]. However, excessive nitrogen fertilization in amaranth cultivation may stimulate excessive vegetative growth, leading to lodging, delayed seed maturation, and accumulation of undesirable nitrate levels in aboveground plant parts [Skwaryło-Bednarz and Nalborczyk 2006, Gimplinger et al. 2007]. Lodging further increases susceptibility to pathogenic diseases and complicates harvesting, thereby negatively affecting final yield size and quality [Skwaryło-Bednarz and Nalborczyk 2006]. Consequently, numerous studies have aimed to identify optimal NPK fertilization strategies for amaranth cultivation. Recent research indicates that seed yield generally responds positively to increasing nitrogen application, although the optimal dose depends on environmental conditions, cultivar, and production objectives. Under diverse agro-climatic conditions, nitrogen rates in the range of 80–120 kg·ha^{-1} have most frequently resulted in the highest seed yields, whereas higher doses tend to enhance vegetative growth at the expense of seed quality [Kozak et al. 2011, Skwaryło-Bednarz et al. 2014, Deryło and Chudzik 2015, Akamine et al. 2021, Mndzebele et al. 2023]. More recent investigations further emphasize that excessive nitrogen fertilization can delay seed maturation and negatively affect quality traits, underscoring the need for balanced and site-specific nutrient management strategies in amaranth cultivation [Akamine et al. 2021, Mndzebele et al. 2023]. These fertilization ranges are consistent with recent findings reported for amaranth cultivated under Central and Eastern European climatic conditions [Tyrus et al. 2024]. The results of the present study indicate that only the interaction between weather conditions during the study years and cultivar significantly affected seed yield (Table 2). In each experimental year, the Aztek cultivar produced a significantly higher seed yield than Rawa. No significant interactions were detected among the other analyzed factors with respect to amaranth seed yield.

Crude fat content and fat yield

One of the basic components of amaranth seeds is crude fat, which contributes significantly to their nutritional and functional value [Krasowska 2022, Malik et al. 2023]. In the present study the factor exerting the strongest influence on crude fat content was cultivar, followed by weather conditions and macronutrient fertilization. The crude fat content in seeds of the studied cultivars showed very low variability, with a coefficient of variation of 4.10% (Table 3). The Aztek accumulated significantly more crude fat than Rawa, with a difference of 2.95 g·kg^{-1} (Table 3), which is consistent with earlier reports [Skwaryło-Bednarz 2012]. The crude fat content recorded in this study was higher than that reported by Tömösközi et al. [2009], but lower than the values reported by Soriano-García and Aguirre-Díaz [2019]. Previous studies have demonstrated that amaranth cultivars differ considerably in seed fat content [Piecyk et al. 2009, Petkova et al. 2019, Azri et al. 2025]. In particular, *A. hypochondriacus* has been shown to exhibit consistently higher fat content than *A. cruentus*, with reported values typically ranging from approximately 4.3 to 7.8% [Bozorov et al. 2018, El Gendy et al. 2018, Azri et al. 2025] which agrees well with the results of the present study. Similar conclusions were also reported by Skwaryło-Bednarz [2010], who emphasized that seed fat content in amaranth depends primarily on species, cultivar, and, to a lesser extent, fertilization level.

Differences in lipid accumulation among cultivars are mainly attributed to genetic variability affecting seed metabolism and storage compound synthesis, as confirmed in large-scale evaluations of amaranth germplasm collections [El Gendy et al. 2018]. Similar conclusions regarding cultivar-dependent variation in seed oil content were reported by Skwaryło-Bednarz [2010], who highlighted the combined role of genotype and agronomic conditions in shaping fat accumulation in amaranth seeds.

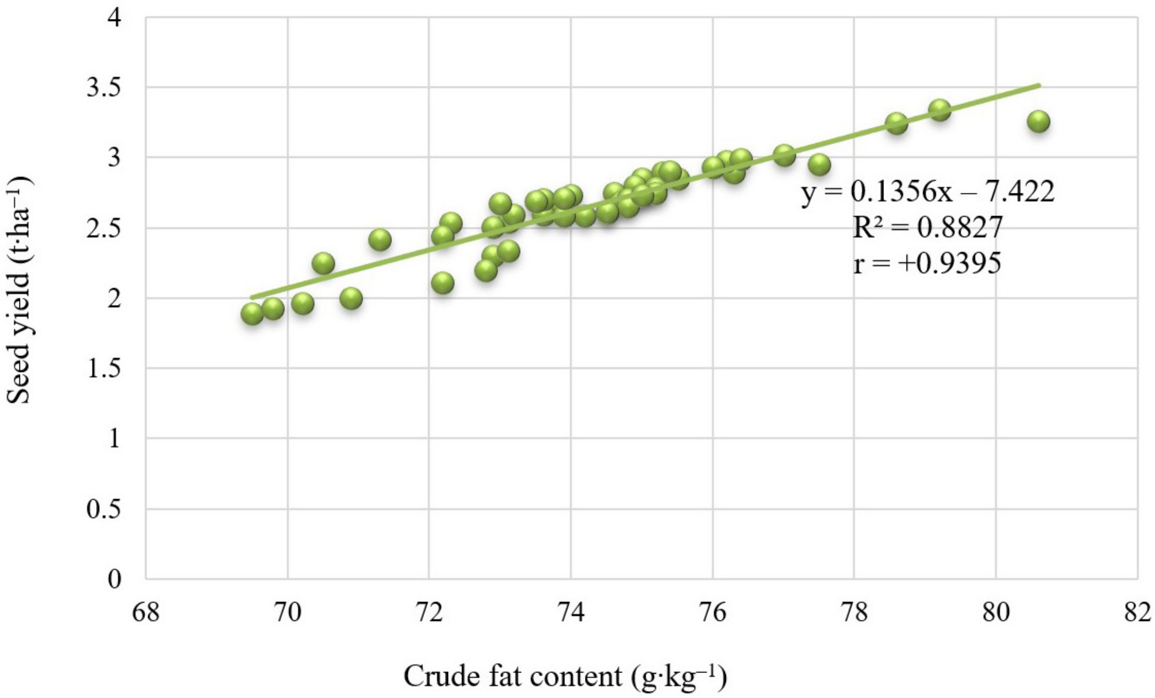
The present study also confirmed a significant influence of weather conditions on crude fat content (Table 3). Seeds harvested in the first and third year contained significantly more crude fat than those obtained in the second year of the study. Most likely, moisture conditions prevailing during the growing seasons favored lipid accumulation in seeds. Comparable relationships between hydrothermal conditions and oil accumulation have been widely documented in oilseed crops, where adequate water supply during seed filling enhances lipid biosynthesis. Kotecki et al. [2007] demonstrated a significant effect of weather conditions on crude fat accumulation in rapeseed. In general, years characterized by favorable rainfall distribution during seed development and maturation are particularly conducive to higher crude fat content in seeds of various crop species.

In contrast to cultivar and weather effects, the level of NPK fertilization exerted the weakest influence on crude fat content. The highest fat content was recorded in seeds from the NPK3 fertilization treatment, whereas the lowest values were observed in the unfertilized control (NPK0). No significant differences in fat content were found between the first and second fertilization levels and the control (Table 3). According to He and Corke [2003], oil content in *Amaranthus* species may vary depending on genetic and environmental factors, while the response to fertilization is often inconsistent, which may explain the relatively weak effect of increasing NPK doses observed in this study.

Fat yield, determined as a function of seed yield and crude fat content, exhibited low variability, with a coefficient of variation of 15.83% (Table 3). Fat yield was influenced more strongly by cultivar than by hydrothermal conditions or NPK fertilization level (Table 3). Genetic factors played a significant role, as the higher seed yield of Aztek resulted in a substantially greater crude fat yield compared with Rawa. On average, Aztek exhibited a 20.1% higher fat yield than Rawa (Table 2). Similar cultivar-related differences in fat yield have been reported for other oilseed and pseudo-cereal crops, emphasizing the importance of genotype selection in improving both yield and quality traits [Bartkowiak-Broda et al. 2005, Sułek et al. 2016].

Fat yield also varied significantly between years, reflecting the strong influence of weather conditions. The highest fat yield was recorded in the first year of the study, whereas the lowest occurred in the second year (Table 3), which corresponds well with earlier findings highlighting the decisive role of hydrothermal factors in oil yield formation

Figure 2. The relationship between amaranth seed yield and crude fat content



[Kotecki et al. 2005]. Fertilization level also significantly affected fat yield (Table 3). The highest values were observed under the NPK3 treatment, while the lowest occurred in the control plots, with a difference of 20.7%. No significant differences in fat yield were found between the first and second fertilization levels and the control (Table 3).

Correlation analysis revealed a strong and significant positive relationship between amaranth seed yield and crude fat content ($r = +0.9395$) – as in Figure 2. Similar strong correlations between seed yield and oil content have been reported for oilseed crops such as rapeseed [Bartkowiak-Broda et al. 2005, Spasibionek 2007]. In the present study, neither crude fat content nor fat yield was affected by interactions among the analyzed factors.

Fatty acid composition of amaranth seed oil

Amaranth seeds contain a considerable amount of fat with a favorable qualitative composition. Due to its fatty acid composition, amaranth oil is classified as a functional food and is rich in biologically active compounds that positively affect human health through hypolipidemic, hypotensive, antioxidant, hepatoprotective, and anti-atherogenic actions [Ratusz and Wirkowska 2006, Moszak et al. 2008, Barrio and Añón 2010]. Similar health-promoting properties of amaranth seed oil, including its functional food potential and bioactive lipid fraction, have been highlighted in recent comprehensive reviews [Malik et al. 2023].

Saturated fatty acids

In amaranth seed oil, eight saturated and seven unsaturated fatty acids were identified (Table 4), which is consistent with recent compositional analyses of amaranth oil obtained from different genotypes and cultivation conditions [Bozorov et al. 2018].

Among saturated fatty acids, lignoceric acid $C_{24:0}$ exhibited moderate variability (CV = 25.29%), while pentadecanoic acid $C_{15:0}$ showed low variability (CV = 14.06%). The other saturated fatty acids were characterized by very low variability, including stearic acid $C_{18:0}$ (3.76%), palmitic acid $C_{16:0}$ (3.85%), behenic acid $C_{22:0}$ (4.79%), myristic acid $C_{14:0}$ (4.86%), arachidic acid $C_{20:0}$ (6.21%), and heptadecanoic acid $C_{17:0}$ (9.18%), as in Table 4.

Hydrothermal conditions significantly affected only the content of behenic acid $C_{22:0}$ (Table 4), although this factor differentiated its concentration to a lesser extent than NPK fertilization and cultivar. The highest content of behenic acid was recorded in the first year of the study and the lowest in the second year – difference of 0.02 percentage points (Table 4). The greatest accumulation of this acid was observed at the third fertilization level (0.31%), while the lowest occurred under NPK0 and NPK1 (0.29%). Seeds of Rawa cultivar contained 0.02 percentage points more behenic acid than Aztek (Table 4).

Table 4. The content of saturated fatty acids in amaranth seed oil (means for factors and years)

Factor	Saturated fatty acids (%)							
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	$C_{24:0}$
NPK0	0.24 d	0.05 c	22.67 a	0.10 b	4.12 b	0.61 a	0.29 c	0.17 a
NPK1	0.25 c	0.06 b	22.55 a	0.10 b	4.16 ab	0.60 a	0.29 c	0.18 a
NPK2	0.26 b	0.06 b	23.00 a	0.11 a	4.23 a	0.60 a	0.30 b	0.20 a
NPK3	0.27 a	0.07 a	22.80 a	0.11 a	4.29 a	0.59 a	0.31 a	0.21 a
$LSD_{0.05}$	0.01	0.01	0.99	0.01	0.16	0.04	0.01	0.05
F value	56.89	7.38	0.57	0.09	3.19	0.42	20.05	1.62
p	***	***	n.s.	**	*	n.s.	***	n.s.
A	0.25 a	0.06 a	23.52 a	0.11 a	4.34 a	0.64 a	0.29 b	0.15 b
R	0.26 a	0.05 b	21.99 b	0.10 b	4.06 b	0.57 b	0.31 a	0.24 a
$LSD_{0.05}$	0.02	0.003	0.25	0.004	0.04	0.01	0.01	0.01
F value	0.4709	39.7794	150.9417	62.3685	162.5563	370.9221	5.7446	281.3377
p	n.s.	***	***	***	***	***	*	***
N	0.25 a	0.06 a	23.00 a	0.10 a	4.176 a	0.60 a	0.30 a	0.19 a
W	0.26 a	0.06 a	22.52 a	0.11 a	4.219 a	0.60 a	0.30 a	0.19 a
$LSD_{0.05}$	0.01	0.01	0.49	0.01	0.093	0.02	0.01	0.03
F value	0.84	3.12	3.79	1.98	0.89	0.42	0.09	0.10
p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2016	0.26 a	0.06 a	22.52 a	0.11 a	4.22 a	0.60 a	**0.31 a**	0.20 a
2017	0.25 a	0.06 a	23.02 a	0.11 a	4.18 a	0.60 a	**0.29 c**	0.18 a
2018	0.26 a	0.06 a	22.74 a	0.11 a	4.20 a	0.60 a	**0.30 b**	0.19 a
$LSD_{0.05}$	0.01	0.01	0.75	0.01	0.14	0.03	**0.01**	0.04
F value	1.12	0.09	1.29	0.09	0.29	0.08	**3.26**	0.59
p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*****	n.s.
CV (%)	4.86	14.06	3.85	9.18	3.76	6.21	4.79	25.29

Legend: NPK0–NPK3 – fertilization levels; A – Aztek cultivar, R – Rawa cultivar; N – narrow row spacing, W – wide row spacing; 2016–2018 – study years; values followed by the same letters do not differ significantly at p ≤ 0.05; *, **, and *** indicate significance at p ≤ 0.05, 0.01, and 0.001, respectively; CV – coefficient of variation

Saturated fatty acids: $C_{14:0}$ – myristic acid; $C_{15:0}$ – pentadecanoic acid; $C_{16:0}$ – palmitic acid; $C_{17:0}$ – heptadecanoic acid; $C_{18:0}$ – stearic acid; $C_{20:0}$ – arachidic acid; $C_{22:0}$ – behenic acid; $C_{24:0}$ – lignoceric acid

The content of myristic acid $C_{14:0}$ was significantly influenced only by fertilization level. Seeds from the control plot contained 0.24% of this acid, and its content increased with successive fertilization levels, reaching 0.27% under NPK3 (Table 4).

Pentadecanoic acid $C_{15:0}$ was more strongly affected by the genetic factor than by fertilization. Seeds of Aztek contained 0.20% more of this acid than Rawa. Although occurring at the lowest concentration among saturated fatty acids, its content was also significantly influenced by fertilization, with the lowest level observed under NPK0 and the highest under NPK3, while NPK1 and NPK2 showed identical values (Table 4).

Palmitic acid $C_{16:0}$ and arachidic acid C20:0 were significantly differentiated only by cultivar (Table 4). The Aztek cultivar contained higher levels of both acids, with differences of 1.53% for palmitic acid and 0.07% for arachidic acid. The dominance of palmitic acid among saturated fatty acids in amaranth oil is consistent with previous reports [He et al. 2002, He and Corke 2003, Nasirpour-Tabrizi et al. 2020, Azri et al. 2025]. Genotype-dependent differences in saturated fatty acid composition have also been demonstrated in evaluations of diverse amaranth germplasm collections [El Gendy et al. 2018, Petkova et al. 2019].

The content of heptadecanoic acid $C_{17:0}$ was influenced more strongly by cultivar than by fertilization, with Aztek containing 0.01 percentage points more than Rawa (Table 4). Its concentration also increased slightly at higher fertilization levels (NPK2 and NPK3) compared with NPK0 and NPK1.

Stearic acid $C_{18:0}$ content depended mainly on cultivar, with Aztek containing 0.28 percentage points more than Rawa (Table 4). The highest content was recorded under NPK3, which did not differ significantly from NPK1 and NPK2, but differed from NPK0. Different trends reported by Azri et al. [2025] under contrasting environments indicate strong genotype × environment interactions governing stearic acid accumulation in amaranth seeds.

Lignoceric acid $C_{24:0}$ was most strongly modified by the genetic factor, with the Rawa cultivar containing 0.09 percentage points more than Aztek (Table 4).

Significant interactions were observed for palmitic acid $C_{16:0}$ and stearic acid $C_{18:0}$, where the interaction between fertilization level and cultivar played a decisive role, confirming the importance of genotype in modifying lipid metabolism under varying agronomic conditions [He and Corke 2003, Skwaryło-Bednarz 2010, El Gendy et al. 2018].

Unsaturated fatty acids

Seven unsaturated fatty acids were identified in amaranth seed oil, with oleic acid $C_{18:1}$ (n-9) and linoleic acid $C_{18:2}$ (n-6) as the dominant components (Table 5). The predominance of these fatty acids is consistent with earlier and recent studies [Ratusz and Wirkowska 2006, Bozorov et al. 2018, El Gendy et al. 2018, Nasirpour-Tabrizi et al. 2020], although interspecific differences within the genus *Amaranthus* have also been reported [Jahaniaval et al. 2000].

Table 5. The content of unsaturated fatty acids in amaranth seed oil (means for factors and years)

Factor	Unsaturated fatty acids (%)						
	$C_{16:1}$ (n-7)	$C_{16:1}$ (n-9)	$C_{18:1}$ (n-7)	$C_{18:1}$ (n-9)	$C_{18:2}$ (n-6)	$C_{18:3}$ (n-3)	$C_{20:1}$ (n-9)
NPK0	0.41 d	0.02 b	1.15 a	29.85 a	39.03 a	1.09 a	0.20 b
NPK1	0.42 c	0.03 ab	1.21 a	29.59 a	39.32 a	1.04 a	0.20 b
NPK2	0.43 b	0.03 ab	1.20 a	29.26 a	39.06 a	1.06 a	0.20 b
NPK3	0.44 a	0.04 a	1.16 a	29.21 a	39.20 a	1.08 a	0.21 a
$LSD_{0.05}$	0.01	0.02	0.07	5.72	4.57	0.24	0.008
F value	17.95	3.39	2.07	0.04	0.01	0.10	4.84
p	***	*	n.s.	n.s.	n.s.	n.s.	**
A	0.42 b	0.02 b	1.13 b	24.46 b	43.16 a	1.25 a	0.20 b
R	0.43 a	0.04 a	1.23 a	34.50 a	35.14 b	0.89 b	0.21 a
$LSD_{0.05}$	0.01	0.006	0.02	0.20	0.15	0.06	0.004
F value	9.32	88.4	102.31	10628.34	12321.31	152.51	32.66
p	**	***	***	***	***	***	***
N	0.42 a	0.03 a	1.17 a	29.41 a	39.02 a	1.07 a	0.20 a
W	0.42 a	0.03 a	1.19 a	29.54 a	39.28 a	1.07 a	0.20 a
$LSD_{0.05}$	0.01	0.01	0.04	2.99	2.38	0.12	0.005
F value	1.14	0.12	1.06	0.01	0.05	0.002	0.11
p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2016	0.43 a	0.04 a	1.20 a	29.56 a	39.23 a	1.07 a	0.21 a
2017	0.41 c	0.03 a	1.16 a	29.42 a	39.07 a	1.04 a	0.20 b
2018	0.42 b	0.03 a	1.18 a	29.46 a	39.17 a	1.10 a	0.20 b
$LSD_{0.05}$	0.01	0.02	0.05	4.45	3.56	0.18	0.007
F value	3.84	1.61	1.72	0.003	0.01	0.33	4.39
p	*	n.s.	n.s.	n.s.	n.s.	n.s.	*
CV (%)	3.49	54.09	5.19	17.07	10.26	19.52	4.19

Legend: NPK0–NPK3 – fertilization levels; A – Aztek cultivar, R – Rawa cultivar; N – narrow row spacing, W – wide row spacing; 2016–2018 – study years; values followed by the same letters do not differ significantly at $p \le 0.05$; *, **, and *** indicate significance at $p \le 0.05$, 0.01, and 0.001, respectively; CV – coefficient of variation

Unsaturated fatty acids: $C_{16:1}$ (n-7) palmitoelaidic acid; $C_{16:1}$ (n-9) palmitoleic acid; $C_{18:1}$ (n-7) elaidic acid; $C_{18:1}$ (n-9) oleic acid; $C_{18:2}$ (n-6) linoleic acid; $C_{18:3}$ (n-3) linolenic acid; $C_{20:1}$ (n-9) eicosenoic acid

Table 6. Fatty acid composition in amaranth seed oil (means for factors)

Factor	Fatty acid composition of cultivated amaranth oil (%)		
	Sum of saturated fatty acids (%)	Sum of unsaturated fatty acids (%)	Ratio of linoleic acid $C_{18:2}$ (n-6) to linolenic acid $C_{18:3}$ (n-3)
NPK0	28.25 a	71.75 a	37.14 a
NPK1	28.20 a	71.80 a	38.04 a
NPK2	28.77 a	71.23 a	37.24 a
NPK3	28.66 a	71.34 a	36.75 a
$LSD_{0.05}$	1.10	1.10	3.74
F value	0.96	0.96	0.30
p	n.s.	n.s.	n.s.
A	29.37 a	70.63 b	34.81 b
R	27.57 b	72.43 a	39.78 a
$LSD_{0.05}$	0.25	0.25	1.31
F value	203.42	203.42	58.55
p	***	***	***
N	28.68 a	71.32 a	37.59 a
W	28.26 a	71.74 a	37.00 a
$LSD_{0.05}$	0.58	0.58	1.96
F value	2.10	2.10	0.36
p	n.s.	n.s.	n.s.
2016	28.27 a	71.73 a	36.94 a
2017	28.69 a	71.31 a	38.17 a
2018	28.45 a	71.55 a	36.77 a
$LSD_{0.05}$	0.87	0.87	2.88
F value	0.68	0.68	0.83
p	n.s.	n.s.	n.s.
CV (%)	3.51	1.40	8.88

Legend: NPK0–NPK3 – fertilization levels; A – Aztek cultivar, R – Rawa cultivar; N – narrow row spacing, W – wide row spacing; 2016–2018 – study years; values followed by the same letters do not differ significantly at $p \leq 0.05$; *, **, and *** indicate significance at $p \leq 0.05$, 0.01, and 0.001, respectively; CV – coefficient of variation

Palmitoleic acid $C_{16:1}$ (n-9) showed strong variability (CV = 54.09%), whereas linolenic acid $C_{18:3}$ (n-3), oleic acid $C_{18:1}$ (n-9), and linoleic acid $C_{18:2}$ (n-6) exhibited low variability (CV = 10.26–19.52%), as in Table 5. The remaining unsaturated fatty acids were characterized by very low coefficients of variation – CV < 10% (Table 5), which is consistent with previous findings [Petkova et al. 2019]. Hydrothermal conditions significantly affected only palmitoelaidic acid $C_{16:1}$ (n-7) and eicosenoic acid $C_{20:1}$ (n-9). The highest contents of both acids were recorded in the first year, while lower or unchanged values were observed in subsequent years (Table 5). Similar sensitivity of minor unsaturated fatty acids to hydrothermal variability has been reported previously [Gamel et al. 2007, Tyrus et al. 2024].

The strongest differentiation of palmitoelaidic acid $C_{16:1}$ (n-7) resulted from NPK fertilization, with the highest content under NPK3 and the lowest under NPK0 (Table 5). Cultivar also significantly affected this acid, with higher concentrations in Rawa. Similar fertilization effects on minor fatty acids have been reported elsewhere [Mndzebele et al. 2023, Tyrus et al. 2024].

Palmitoleic acid $C_{16:1}$ (n-9), elaidic acid $C_{18:1}$ (n-7), oleic acid $C_{18:1}$ (n-9), linoleic acid $C_{18:2}$ (n-6), linolenic acid $C_{18:3}$ (n-3), and eicosenoic acid $C_{20:1}$ (n-9) were primarily influenced by cultivar (Table 5). Oleic acid content was substantially higher in Rawa, exceeding values reported for several amaranth genotypes grown under different conditions [Jahaniaval et al. 2000, León-Camacho et al. 2001, Escudero et al. 2004]. In contrast, Aztek exhibited higher concentrations of linoleic and linolenic acids (Table 5). Such cultivar-dependent variation is attributable to genetic differences in lipid biosynthesis pathways, including Δ9 and Δ12 desaturase activities [Cerone et al. 2022].

A significant interaction between row spacing and cultivar was observed for linoleic acid $C_{18:2}$ content (Table 2), indicating that plant spatial arrangement may modulate seed quality traits in a genotype-dependent manner, as reported previously [Gamel et al. 2007, Petkova et al. 2019].

Fatty acid ratios and nutritional implications

Coefficients of variation for the sum of saturated and unsaturated fatty acids and for the linoleic to linolenic acid ratio were very low (Table 6), indicating stable proportions of major fatty acid groups, consistent with earlier studies [Petkova et al. 2019, Baraniak and Kania-Dobrowolska 2022].

Cultivar was the main factor differentiating the sums of saturated and unsaturated fatty acids and the n-6/n-3 ratio (Table 6). The Aztek cultivar contained a higher proportion of saturated fatty acids, while Rawa showed a higher proportion of unsaturated fatty acids. The saturated-to-unsaturated fatty acid ratio in both cultivars was close to 1:3, in agreement with previous reports [Gontarczyk 1996, He et al. 2002, Januszewska-Jóźwiak and Synowiecki 2008, El Gendy et al. 2018].

The n-6/n-3 ratio was higher in Rawa (39.78:1) than in Aztek (34.81:1) – as in Table 6, confirming that amaranth oil is generally characterized by a high n-6/n-3 ratio, as also reported in food composition databases [USDA FoodData Central 2023]. Nevertheless, genotype-dependent variability has been widely documented [El Gendy et al. 2018, Petkova et al. 2019]. From a nutritional perspective, these differences are highly relevant, as emphasized by Jan et al. [2023]. Although the observed ratios exceed recommended dietary values, cultivar-dependent variation and significant genotype × agronomic interactions indicate potential for improving fatty acid balance through targeted selection and cultivation practices [Simopoulos 2016, Calder 2017].

CONCLUSIONS

The three-year field experiment demonstrated that seed yield, crude fat content, fat yield, and fatty acid composition of amaranth cultivated under temperate climatic conditions are determined by the combined effects of genetic and agrotechnical factors. Among the analyzed variables, cultivar proved to be the primary factor differentiating both yield potential and oil quality traits. The Aztek cultivar was characterized by higher seed yield and fat yield, whereas the Rawa cultivar exhibited a more favorable fatty acid composition, particularly in terms of the degree of unsaturation.

Row spacing significantly influenced plant density and yield structure. Wider row spacing (55 cm) promoted higher seed mass per plant and modified fatty acid composition in a cultivar-dependent manner, indicating the importance of adjusting stand architecture to genotype. Increasing NPK fertilization level enhanced seed yield and fat yield; however, its effect on fatty acid composition was limited and depended on cultivar. Significant interactions between fertilization level and cultivar, particularly for palmitic (C16:0) and stearic (C18:0) acids, confirm genotype-specific responses to nutrient supply. Weather conditions markedly affected yield formation and fat accumulation, but did not alter the overall direction of cultivar and agrotechnical effects.

From a practical perspective, the results indicate that effective amaranth cultivation under temperate climate conditions should combine appropriate cultivar selection with optimized fertilization level and row spacing. In particular, the integration of moderate to high mineral fertilization with wider row spacing may contribute to increased seed productivity while maintaining favorable oil quality traits. The findings also indicate that cultivar-specific responses should be taken into account when formulating agrotechnical recommendations.

Further research should focus on refining fertilization strategies and stand management under variable climatic conditions, with particular emphasis on their effects on seed yield, crude fat content, and fatty acid composition. Improved understanding of genotype × environment × management interactions may contribute to more precise optimization of agrotechnical practices aimed at maintaining high yield levels while ensuring stable and desirable oil quality traits of amaranth seeds.

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REFERENCES

Achremowicz, B.,Ooglińska, A., Haber, T. et al. (2015). Ogólna charakterystyka i technologiczne wykorzystanie nasion szarłatu. Część I. Ogólna charakterystyka szarłatu [General characteristics and technological use of amaranth seeds. Part I. General characteristics of amaranth]. Post. Tech. Przetw. Spoż., 1, 118–125.

Akamine, H., Ohshiro, M., Hossain, M.A. (2021). Effects of N, P and K fertilizers on edible amaranth (*Amaranthus* spp.) grown on the red soil of Okinawa. Appl. Ecol. Environ. Res., 19(3), 2333–2346. https://doi.org/10.15666/aeer/1903_23332346

Azri, A., Aydi, S.S., Aydi, S. et al.(2025). Nutritional and bioactive lipid composition of *Amaranthus* seeds grown in varied agro-climatic conditions in France. Agronomy, 15, 672. https://doi.org/10.3390/agronomy15030672

Baraniak, J., Kania-Dobrowolska, M. (2022). The dual nature of amaranth-functional food and potential medicine. Foods, 11(4), 618. https://doi.org/10.3390/foods11040618

Barrio, D.A, Añón, M.C. (2010). Potential antitumor properties of a protein isolate obtained from the seeds of *Amaranthus mantegazzianus*. Eur. J. Nutr., 49(2), 73–82. https://doi.org/10.1007/s00394-009-0051-9

Bartkowiak-Broda, I., Wałkowski, T., Ogrodowczyk, M. (2005). Przyrodnicze i agrotechniczne możliwości kształtowania jakości nasion rzepaku [Natural and agrotechnical possibilities of shaping the quality of rapeseed seeds]. Pam. Puł., 139, 7–19.

Bielski, S., Szwejkowska, B. (2015). Influence of some agro-technical treatments on the development and yields of amaranth. Bulg. J. Agric. Sci., 21(4), 909–915. Available: http://www.agrojournal.org/21/04-34.pdf [date of access: 11.02.2025].

Bozorov, S.S., Berdiev, N.S., Ishimov, U.J. et al. (2018). Chemical composition and biological activity of seed oil of amaranth varieties. Nova Biotechnol. Chim., 17(1), 66–73. https://doi.org/10.2478/nbec-2018-0007

Calder, P.C. (2017). Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem. Soc. Trans., 15, 45(5), 1105–1115. https://doi.org/10.1042/BST20160474

Cerone, M., Smith, T.K. (2022). Desaturases: Structural and mechanistic insights into the biosynthesis of unsaturated fatty acids. IUBMB Life, 74(11), 1036–1051. https://doi.org/10.1002/iub.2671

Deryło, S., Chudzik, Ł. (2015). Planowanie szarłatu uprawnego (*Amaranthus hypochondriacus* L. Thell.) w warunkach zróżnicowanej przedsiewnej uprawy roli i pielęgnacji roślin [Cultivation of grain amaranth (*Amaranthus hypochondriacus* L. Thell.) under conditions of varied pre-sowing soil tillage and plant care]. Annales UMCS, sec. E, Agricultura, 70(2), 55–65.

El Gendy, A.N.G., Tavarini, S., Conte, G. et al. (2018). Yield and qualitative characterisation of seeds of *Amaranthus hypochondriacus* L. and *Amaranthus cruentus* L. grown in central Italy. Ital. J. Agron., 13(1), 993. https://doi.org/10.4081/ija.2017.993

Escudero, N.L., de Arellano, M.L., Luco, J.M. et al. (2004). Comparison of the chemical composition and nutritional value of *Amaranthus cruentus* flour and its protein concentrate. Plant Foods Hum. Nutr., 59(1), 15–21. https://doi.org/10.1007/s11130-004-0033-3

Gamel, T.H., Mesallam, A.S., Damir, A.A. et al. (2007). Characteristics of amaranth seed oil. Int. J. Food Prop., 10(1), 1–13. https://doi.org/10.1080/10942910600596442

Gimplinger, D.M., Dobos, G., Schönlechner, R. et al. (2007). Yield and quality of grain amaranth (*Amaranthus* sp.) in Eastern Austria. Plant Soil Environ., 53(3), 105-112.

Gontarczyk, M. (1996). Szarłat uprawny – *Amaranthus* spp. [Grain amaranth – *Amaranthus* spp.]. In: Nowe rośliny uprawne na cele spożywcze, przemysłowe i jako odnawialne źródła energii [New crop plants for food, industrial purposes, and as renewable energy sources], Nalborczyk, E. (ed.). Wyd. SGGW, Warszawa, 21–43.

Graham, M.W. (2010). Grain amaranth production and effects of soil amendments in Uganda. Graduate Theses and Dissertations, Paper 11462. Iowa State University, USA. https://doi.org/10.31274/etd-180810-102

Grobelnik Mlakar, S., Bavec, M., Jakop, M. et al. (2012). The effect of drought occurring at different growth stages on productivity of grain amaranth *Amaranthus cruentus* G6. J. Life Sci., 6(3), 283–286.

Haliniarz, M., Bujak, K., Gawęda, D. et al. (2013). Reakcja pszenicy jarej na uproszczenia w uprawie roli i zróżnicowany poziom nawożenia mineralnego [Response of spring wheat to simplifications in soil cultivation and varied levels of mineral fertilization]. Acta Sci. Pol, Agricultura (Agronomia), 12(3), 13–24.

He, H.P., Cai, Y., Sun, M. et al. (2002). Extraction and purification of squalene from amaranthus grain. J. Agric. Food Chem., 50(2), 368–372. https://doi.org/10.1021/jf010918p

He, H.P., Corke, H. (2003). Oil and squalene in amaranthus grain and leaf. J. Agric. Food Chem., 51(27), 7913–7920. https://doi.org/10.1021/jf034775p

Jahaniaval, F., Kakuda, Y., Marcone, M.F. (2000). Fatty acid and triacylglycerol compositions of seed oils of five amaranthus accessions and their comparison to other oils. J. Am. Oil Chem. Soc., 77(8), 847–852. Available: http://lib3.dss.go.th/fulltext/Journal/J.AOCS/J.AOCS/2000/no.8/aug2000vol77,no8,p847-852.pdf [date of access: 23.04.2025].

Jan, N., Hussain, S., Naseer, B. et al. (2023). Amaranth and quinoa as potential nutraceuticals: a review of anti-nutritional factors, health benefits and their applications in food, medicinal and cosmetic sectors. Food Chem. X., 18, 100687. https://doi.org/10.1016/j.fochx.2023.100687

Januszewska-Jóźwiak, K., Synowiecki, J. (2008). Charakterystyka i przydatność składników szarłatu w biotechnologii żywności. [Characteristics and suitability of amaranth components in food biotechnology]. Biotechnologia, 3(82), 89–102.

Jendrzejczak, E., Śmigerska, K. (2014). Impact of presowing soaking of amaranth seeds in solutions of growth regulators. Part II. Effect of treatments processing seed material on growth, development and yield of amaranth cv. Rawa. Acta Sci. Pol., Agricultura, 13(4), 53–64.

Kotecki, A., Kozak, M., Malarz, W. (2005). Wpływ zróżnicowanej technologii uprawy na rozwój i plonowanie odmian rzepaku ozimego [The influence of diversified cultivation technology on the growth and yield of winter rapeseed varieties]. Rośliny Oleist – Oilseed Crops, 26(1), 111–124.

Kotecki, A., Malarz, W., Kozak, M. et al. (2007). Wpływ rozmieszczenia roślin w łanie na rozwój i plonowanie mieszańcowych i populacyjnych odmian rzepaku. Część II. Skład chemiczny i wartość energetyczna [The influence of plant distribution in the stand on the growth and yield of hybrid and population varieties of rapeseed. Part II. Chemical composition and energy value]. Zesz. Nauk. UP Wroc. 553, Rolnictwo, 90, 41–65.

Kozak, M., Malarz, W., Kotecki, A. et al. (2011). Wpływ zróżnicowanego nawożenia azotem na rozwój, plonowanie i skład chemiczny nasion amarantusa uprawnego [The effect of varied nitrogen fertilization on the growth, yield, and chemical composition of cultivated amaranth seeds]. Zesz. Nauk. UP Wroc. 581, Rolnictwo, 98, 79–94.

Krasowska, P. (2022). Effect of different NPK fertilisation and cultivar on the size, quality and healthiness of amaranth seed yield. Ph.D. Thesis. Typescript, University of Life Sciences in Lublin, Poland.

León-Camacho, M., Garcia-Gonzalez, D.L., Aparicio, R. (2001). A detailed and comprehensive study of amaranth (*Amaranthus cruentus* L.) oil fatty profile. Eur. Food Res. Technol., 213, 349–355. https://doi.org/10.1007/s002170100340

Malik, M., Sindhu, R., Dhull, S.B. et al. (2023). Nutritional composition, functionality, and processing technologies for amaranth. J. Food Process. Preserv., 1753029. https://doi.org/10.1155/2023/1753029

Managa, G.M., Nemadodzi, L.E. (2023). Comparison of agronomic parameters and nutritional composition on red and green amaranth species grown in open field versus greenhouse environment. Agriculture, 13, 685. https://doi.org/10.3390/agriculture13030685

Martínez-Núñez, M., Ruiz-Rivas, M., Vera-Hernández, P.F. et al. (2019). The phenological growth stages of different amaranth species grown in restricted spaces based in BBCH code. S. Afr. J. Bot., 124, 436-443. https://doi.org/10.1016/j.sajb.2019.05.035

Mndzebele, B., Ncube, B., Fessehazion, M., et al. (2023). Assessment of the effects of NPK fertilizer on edible yield and agro-biological parameters in a cowpea–amaranth intercrop. Front. Sustain. Food Syst., 7, 1210984. https://doi.org/10.3389/fsufs.2023.1210984

Moszak, M., Zawada, A., Grzymisławski, M. (2008). Właściwości oraz zastosowanie oleju rzepakowego i oleju z amarantusa w leczeniu zaburzeń metabolicznych związanych z otyłością [Properties and applications of rapeseed oil and amaranth oil in the treatment of metabolic disorders associated with obesity]. Forum Zaburzeń Metab., 9(2), 53–64.

Nasirpour-Tabrizi, P., Azadmard-Damirchi, S., Hesari, J. et al. (2020). Amaranth seed oil composition. Nutritional Value of Amaranth. IntechOpen. Available: http://dx.doi.org/10.5772/intechopen.91381 [date of access: 23.06.2025].

Olofintoye, J.A.T., Abayomi, Y.A., Olugbemi, O. (2015). Yield response of grain amaranth (*Amaranthus cruentus* L.) varieties to varying planting density and soil amendment. Afr. J. Agric. Res., 10(21), 2218–2225. https://doi.org/10.5897/AJAR2015.9746

Olufolaji, A.O., Odeleye, F.O., Ojo, O.D. (2010). Effect of soil moisture stress on the emergence, establishment and productivity of amaranthus (*Amaranthus cruentus* L.). Agric. Biol. J. N. Am., 1(6), 1170–1180. Hhttps://doi.org/10.5251/abjna.2010.1.6.1169.1181

Petkova, Z.Y., Antova, G.A., Angelova-Romova, M.I. et al. (2019). A comparative study on chemical and lipid composition of amaranth seeds with different origin. Bulgar. Chem. Commun., 51, D, 262–267.

Piecyk, M., Worobiej, E., Rębiś, M. et al. (2009). Content and characteristics of nutrients in amaranth products. Bromat. Chem. Toksykol., 42(2), 147–153.

PN-79R-65950. Materiał siewny. Metody badania nasion [Seed material. Methods of seed testing.].

Pusz, W., Pląskowska, E., Yildirim, I. et al. (2015). Fungi occurring on the plants of the genus *Amaranthus* L. Turk. J. Bot., 39, 147–161. https://doi.org 10.3906/bot-1403-106

Ratusz, K., Wirkowska, M. (2006). Charakterystyka nasion i lipidów amarantusa [Characteristics of amaranth seeds and lipids.]. Rośl. Oleiste – Oilseed Crops, 27(2), Suppl., 243–250.

Sayed-Ahmad, B., Urrutigoïty, M., Hijazi, A. et al. (2022). Amaranth oilseed composition and cosmetic applications. Separations, 9(7), 181. https://doi.org/10.3390/separations9070181

Schafleitner, R., Lin, Y.-P., Dinssa, F.F., et al. (2022). The World Vegetable Center Amaranthus germplasm collection: core collection development and evaluation of agronomic and nutritional traits. Crop Sci., 62(3), 1240–1256. https://doi.org/10.1002/csc2.20715

Simopoulos, A.P. (2016). An increase in the Omega-6/Omega-3 fatty acid ratio increases the risk for obesity. Nutrients, 8(3), 128. https://doi.org/10.3390/nu8030128

Skwaryło-Bednarz, B. (2010). Zawartość tłuszczu oraz tokoferoli w nasionach krajowych odmian szarłatu (*Amaranthus cruentus* L.) w warunkach zróżnicowanego nawożenia makroelementami [Fat and tocopherol content in seeds of domestic amaranth (*Amaranthus cruentus* L.) cultivars under differentiated macronutrient fertilization conditions.]. Acta Agrophys., 15(2), 409–415.

Skwaryło-Bednarz, B. (2012). Assessment of content of fat and tocopherols in seeds of amaranthus in relation to diversified fertilization with macroelements. Ecol. Chem. Eng. S, 19(2), 273–279. https://doi.org/10.2478/v10216-011-0021-z

Skwaryło-Bednarz, B., Brodowska, M.S., Brodowski, R. (2011). Evaluating the influence of varied NPK fertilization on yielding and microelements contents at amaranth (*Amaranthus cruentus* L.) depending on its cultivar and plant spacing. Acta Sci. Pol. Hortorum Cultus, 10(4), 245–261.

Skwaryło-Bednarz, B., Brodowska, M.S., Onuch, J. et al. (2014). Plonowanie krajowych odmian szarłatu (*Amaranthus cruentus* L.) w warunkach zróżnicowanego nawożenia makroelementami [Yield performance of domestic amaranth (*Amaranthus cruentus* L.) cultivars under differentiated macronutrient fertilization conditions.]. Ann. UMCS, sec. E, Agricultura, 69(2), 37–43.

Skwaryło-Bednarz, B., Nalborczyk, E. (2006). Uprawa i wykorzystanie amarantusa [Cultivation and utilization of amaranth.]. Wieś Jutra, 4(93), 52–55.

Skwaryło-Bednarz, B., Stępniak, P., Jamiołkowska, A. et al. (2020). Amaranth seeds as a source of nutrients and bioactive substances in human diet. Acta Sci. Pol. Hortorum Cultus, 19(6), 153–164. https://doi.org/10.24326/asphc.2020.6.13

Soriano-García, M., Aguirre-Díaz, I.S. (2019). Nutritional functional value and therapeutic utilization of amaranth. In: V.Y. Waisundara, Nutritional value of amaranth. IntechOpen. https://doi.org/10.5772/intechopen.86897

Spasibionek, S. (2007). Postępy w badaniach nad genetyką i hodowlą rzepaku (*Brassica napus* L.) [Advances in research on rapeseed genetics and breeding (*Brassica napus* L.)]. Rośliny Oleiste – Oilseed Crops, 28, 68–73.

Sułek, A., Podolska, G., Jaśkiewicz, B. (2016). Plonowanie i zdrowotność dwóch podgatunków pszenicy w zależności od udziału zbóż w strukturze zasiewów w warunkach integrowanej produkcji [Yield and health of two wheat subspecies depending on the share of cereals in the crop structure under integrated production conditions]. Pol. J. Agron., 27, 118–125.

Tömösközi, S. et al. (2009). Comparative study of composition and technological quality of amaranth. I. Gross chemical composition, amino acid and mineral content. Acta Aliment., 38, 341–347. https://doi.org/10.1556/AAlim.38.2009.3.8

Tyrus, M., Lykhochvor, V., Hnativ, P. et al. (2024). The effect of mineral fertilizer doses on amaranth grain quality in the wet climate of Western Ukraine. J. Elementol., 29(3), 635–646. https://doi.org/10.5601/jelem.2024.29.2.3359

USDA FoodData Central. 2023. Amaranth grain. U.S. Department of Agriculture. https://fdc.nal.usda.gov

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DETECTION AND MOLECULAR CHARACTERISATION OF THE *Orthotospovirus iridimaculaflavi* N GENE OF THE ONION IN POLAND

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ABSTRACT

Orthotospovirus iridimaculaflavi (iris yellow spot orthotospovirus, IYSV) is one of the most important viral pathogens affecting onions worldwide. The virus is predominantly transmitted by onion thrips and can cause significant losses in cultivated fields. In this study, the identification and molecular characterisation of IYSV isolates originating from onion crops in Poland were carried out. Symptomatic onion plants (*Allium cepa* L.) were observed in Kujawsko-Pomorskie, Lubelskie, and Wielkopolskie regions of Poland. The plants were characterised by various disease symptoms from mild to severe, resulting in deformation and growth reduction. A total of 40 onion samples were collected, and the presence of IYSV was confirmed in three of them. Moreover, the occurrence of *Potyvirus cepae* (onion yellow dwarf virus, OYDV) in single and mixed infections with IYSV was observed. The sequences of IYSV obtained in this study were compared with the sequences retrieved from the GenBank database, and the phylogenetic analysis was subsequently conducted. The maximum-likelihood reconstruction revealed that the Polish isolates mainly grouped with isolates originating from Serbia. To our knowledge, it is the first report of IYSV infecting onions in Poland.

Keywords: iris yellow spot orthotospovirus, IYSV, *Allium cepa* L., RT-PCR, phylogenetic analysis

INTRODUCTION

The onion (*Allium cepa* L.) is one of the valuable vegetables that holds the top vegetable crops cultivated in Poland. Based on the latest statistics released by the Central Statistical Office in 2023, the area of onion cultivation in Poland covered 22,133 hectares, producing approximately 0.6 million tons.

Onions can be infected by numerous pathogens, including bacteria, fungi, phytoplasmas, and viruses, which threaten its cultivation, growth, and production [Schwartz and Mohan 2016]. In previous years, the most common virus infecting onion crops in Poland was *Potyvirus cepae* (onion yellow dwarf potyvirus, OYDV), belonging to the *Potyvirus* genus [Taberska et al. 2021]. This virus causes streaking, yellowing, and bending of leaves. Another common virus in onion cultivation worldwide is *Orthotospovirus iridimaculaflavi* (iris yellow spot orthotospovirus, IYSV). IYSV is a member of the Tospoviridae family, within the order *Elliovirales* and the genus *Orthotospovirus*. The virus was first observed in onion fields in Brazil in 1981 [De Avila et al. 1981] and on iris and leek in the Netherlands [Derks and Lemmers 1996, Cortês et al. 1998]. IYSV is widespread in most regions of the world where *Allium* species are cultivated. Its presence has been documented across several continents, including: North America, South America, Europe, Asia, Africa, and Oceania [Tabassum et al. 2021]. In Poland, it was first detected in 2005 on chrysanthemum crops [Balukiewicz and Kryczyński 2005].

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IYSV infects a variety of hosts, including onions, garlic, chives, leeks, several ornamental plants such as chrysanthemums, and weeds [Bag et al. 2015, Balukiewicz and Kryczyński 2005, Tabassum et al. 2021]. The virus is efficiently transmitted by onion thrips (*Thrips tabaci* Lindeman) and with less efficiency by tobacco thrips (*Frankliniella fusca* Hinds) [Srinivasan et al. 2012]. In recent years, the virus has caused significant economic losses in onion cultivation in the United States and several other countries, which has been associated with the increasing incidence of thrips in these regions [Shin and Rho 2014, Bag et al. 2015, Tabassum et al. 2021].

The virus induces morphological alterations characterised by yellow to bleached-white rhomboid lesions on the leaves, reduces bulb development, and disrupts photosynthetic activity. These effects collectively lead to a significant decrease in crop yield and create entry points for other pathogens and secondary colonizers [Nischwitz et al. 2007]. IYSV particles are helical, measuring 80–120 nm, and consist of RNA, protein, glycoprotein, and lipids [Bag et al. 2015].

The IYSV genome consists of three single-stranded RNAs: small RNA (S RNA), medium RNA (M RNA), and large RNA (L RNA) [Clabbers et al. 2014]. The L RNA is 8880 nucleotides long and contains a single open reading frame (ORF) of 8621 nucleotides in the viral complementary (vc) strand, potentially coding for the viral RNA-dependent RNA polymerase (RdRp). The M RNA is 4821 nucleotides long with two ORFs in ambisense arrangement, potentially coding two glycoproteins (GN and GC) and a non-structural protein (NSm). The S RNA is 3105 nucleotides long and encodes for two non-overlapping ORFs in an ambisense arrangement: the nucleocapsid (N) and the non-structural (NSs) proteins. The S RNA structures of viruses representing the *Orthotospovirus* genus have been found to interact with the viral proteins N and NSs, likely involving the binding of NSs to RNA [Clabbers et al. 2014].

IYSV has not been reported in onion cultivation in Poland so far. However, in recent years, symptoms resembling those caused by orthotospoviruses have been observed in onion crops. Therefore, in this study we analysed the occurrence of IYSV and OYDV in onion crops. Moreover, the phylogenetic relationships of IYSV isolates were established.

MATERIALS AND METHODS

Plant material

During two consecutive vegetative seasons (2023 and 2024), a survey of onion crops in Poland was conducted. A total of 40 leaf samples, including 6 from Lubelskie (50.909722°N; 22.834444°E), 24 from Wielkopolskie (52.1194°N; 16.7886°E), and 10 from Kujawsko-Pomorskie (52.849444°N; 18.476389°E) regions, were collected. The samples were stored at –80 °C until further analyses.

Total RNA extraction

Total RNA from the symptomatic and asymptomatic leaf samples (100 mg of leaf tissue) was extracted using the TRI Reagent procedure (Thermo Fisher Scientific, Wilmington, DE, USA) and dissolved in 30 µl of sterile water. Purity and concentration of obtained RNAs were measured by using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

RT-PCR (reverse transcription polymerase chain reaction)

First-strand complementary DNA (cDNA) synthesis was done using SuperScript III (One-Step RT-PCR System with Platinum™ Taq DNA Polymerase, Thermo Fisher Scientific, USA). The samples were tested for the presence of OYDV and IYSV using previously published primers (Table 1) [Manglli et al. 2014, Shin and Rho 2014]. The reaction mixture consisted of 22 µl of sterile water, 25 µl of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA), 2 µl of forward and reverse primer mixtures, and 1 µl of template cDNA. The PCR reaction was carried out in a thermal profile as previously described [Manglli et al. 2014, Shin and Rho 2014].

To obtain full-length nucleocapsid (N) protein gene sequences of IYSV, the new primer pair (Table 1) was designed using OligoAnalyzer, based on the set of IYSV sequences retrieved from GenBank. The PCR reaction was optimized using designed primers in a T-Professional thermocycler with a temperature gradient block (Biometra, Germany) with a programmed temperature gradient from 45°C to 55°C. The reaction mixture was prepared as described above and followed the manufacturer's protocol.

Obtained PCR products were separated in 1% agarose gel with Midori Green dye (Nippon Genetics Europe, Düren, Germany) with GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, USA). Results were observed with a UV transilluminator (Vilber, France). The PCR products of appropriate size were purified using NucleoSpin®Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany), according to the manufacturer's protocol. After PCR, the products were sequenced using the Sanger method by an external company (Genomed S.A., Warsaw, Poland) to verify their specificity.

Table 1. Primers used for the detection of IYSV and OYDV in RT-PCR reactions [Manglli et al. 2014, Shin and Rho 2014]

Primers	Sequence 5'– 3'	Product size (bp)	Melting temperature (°C)	Reference
IYSV_N30 IYSV_C40	GCTCGTAAGTTGAGAATCTGC TGGACATTCAGGAGGTTG	307	50	Shin and Rho 2014
IYSV-NgeneR IYSV-NgeneF	CTCTTAAACACATTTAACAAGCA TAAAACAAACATTCAAACAA	1069	53	designed in this study
OYDV-NibCPF1 OYDV-NibCPR1	CATCCAGATCACGAGGGAAT TGTGGCATTTCGGTATTCAA	987	52	Manglli et al. 2014

Selective pressure analysis

IYSV nucleocapsid (N) protein gene sequences of the Polish isolates, consisting of 822 nucleotides, were examined, edited and compiled using BioEdit software [Hall 1999]. Subsequently, the obtained sequences were compared with 46 other IYSV nucleocapsid (N) protein gene sequences available in the GenBank database. The collected sequences were aligned using the MUSCLE algorithm as implemented in MEGA X software [Kumar et al. 2018].

Selective pressure analysis in plant viruses helps to understand how viruses evolve and adapt to their environment [Hasiów-Jaroszewska et al. 2014, LaTourrette and Garcia-Ruiz 2022]. Our analysis of selective pressures was based on ω value estimated for each codon in the nucleotide sequence alignment. The selective pressure affecting individual codons was then analysed using the Datamonkey Adaptive Evolution Server [Weaver et al. 2018] with four algorithms: Mixed Effects Model of Evolution (MEME), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian Approximation (FUBAR), and Single-Likelihood Ancestor Counting (SLAC). The selective pressures were quantified by estimating the ratio of non-synonymous (d_N) to synonymous (d_S) substitution rates ($\omega = d_N/d_S$), with $\omega > 1$ indicating diversifying selection, $\omega = 1$ neutral selection and $\omega < 1$ purifying selection [Lemey et al. 2009]. Thresholds for statistical significance were set at $p < 0.05$ for MEME, $p < 0.1$ for FEL and SLAC, and a posterior probability > 0.9 for FUBAR, in line with the Bayesian approach.

Recombination analysis

Prior to phylogenetic analysis, the potential recombination events in the analysed IYSV population were examined by the Recombination Detection Program 4. The analysis was performed by seven different detection algorithms: RDP, Chimaera, BootScan, 3 Seq, GENECONV, MaxChi, SiScan [Martin et al. 2015]. Default parameters and a p-value threshold of 0.05 were used. The recombination events were considered statistically significant if five or more methods had a $p < 0.05$. To confirm the results, an analysis of recombinants was also conducted using the SplitsTree program [Huson and Bryant 2006].

Sequence analysis

Phylogenetic analysis was carried out using the maximum-likelihood algorithm implemented in MEGA X with 49 full-length sequences of the IYSV nucleocapsid (N) protein gene (Table 2). *Orthotospovirus arachinecrosis* (peanut bud necrosis virus, PBNV, accession number: MG913145) was selected as the outgroup. The Tamura-3 nucleotide substitution model with gamma distribution (T92 + G) [Kumar et al. 2018] was applied, along with 1000 random pseudoreplicates. Sequence identity matrices were prepared using BioEdit [Hall 1999] and Sequence Demarcation Tool Version 1.2 (SDTv1.2) [Muhire et al. 2014].

Table 2. Host plant, geographic origin, collection date, and GenBank accession numbers of IYSV isolates used in phylogenetic analysis

Accession no.	Location	Host	Collection date
EU477515	New Zealand	*A. cepa*	2007
OP357939	Germany	*A. ampeloprasum*	–
FJ785835	Greece	*A. cepa*	2008
AY345227	Australia	*A. cepa*	–
MG065699	Iran	*A. porrum*	2015
MF420346	Iran	*A. ampeloprasum*	2015
MF431883	Iran	*A. cepa*	2015
HQ148173	Iran	*A. cepa*	2009
MF431884	Iran	*A. cepa*	2016
HQ148174	Iran	*A. cepa*	2009
MF420345	Iran	*A. cepa*	2015
DQ233479	USA	*A. cepa*	–
AB871456	Japan	*A. cepa*	–
AB505813	Japan	*A. chinense*	2008
AB121025	Japan	*E. russellianum*	–
AB871444	Japan	*A. cepa*	–
AB871438	Japan	*A. cepa*	–
AB180922	Japan	*Alstroemeria*	–
AB180920	Japan	*A. cepa*	–
AB871451	Japan	*A. cepa*	–
AB180918	Japan	*E. russellianum*	–
AB871455	Japan	*A. cepa*	–
GU901211	Sri Lanka	*A. porrum*	2009
FJ514257	USA	*A. sativum*	2008
DQ233476	USA	*A. cepa*	–
AY345226	Australia	*A. cepa*	–
AF271219	Israel	*E. russellianum*	–
DQ270004	India	*A. cepa*	–
KJ868797	India	*A. cepa*	2014
EU310292	India	*A. cepa*	–
EU310280	India	*A. cepa*	–
EU310270	India	*A. cepa*	–
EU310300	India	*A. cepa*	–
KC161369	Egypt	*A. cepa*	2012
AF067070	Brazil	*A. cepa*	–
JX861126	Bosnia and Herzegovina	*A. cepa*	2012
JQ973065	USA	*A. cepa*	2011
MH172159	Pakistan	*A. sativum*	2017
KT272884	Serbia	*A. cepa*	2014
KT272879	Serbia	*A. cepa*	2014
JQ973066	USA	*A. cepa*	2009
EU727180	Serbia	*A. cepa*	2007
EU287943	Canada	*A. cepa*	–
MH172160	Pakistan	*A. cepa*	2017
KX147286	USA	*A. cepa*	2015
MF359019	Zimbabwe	*A. sativum*	2015

Note: – data not available

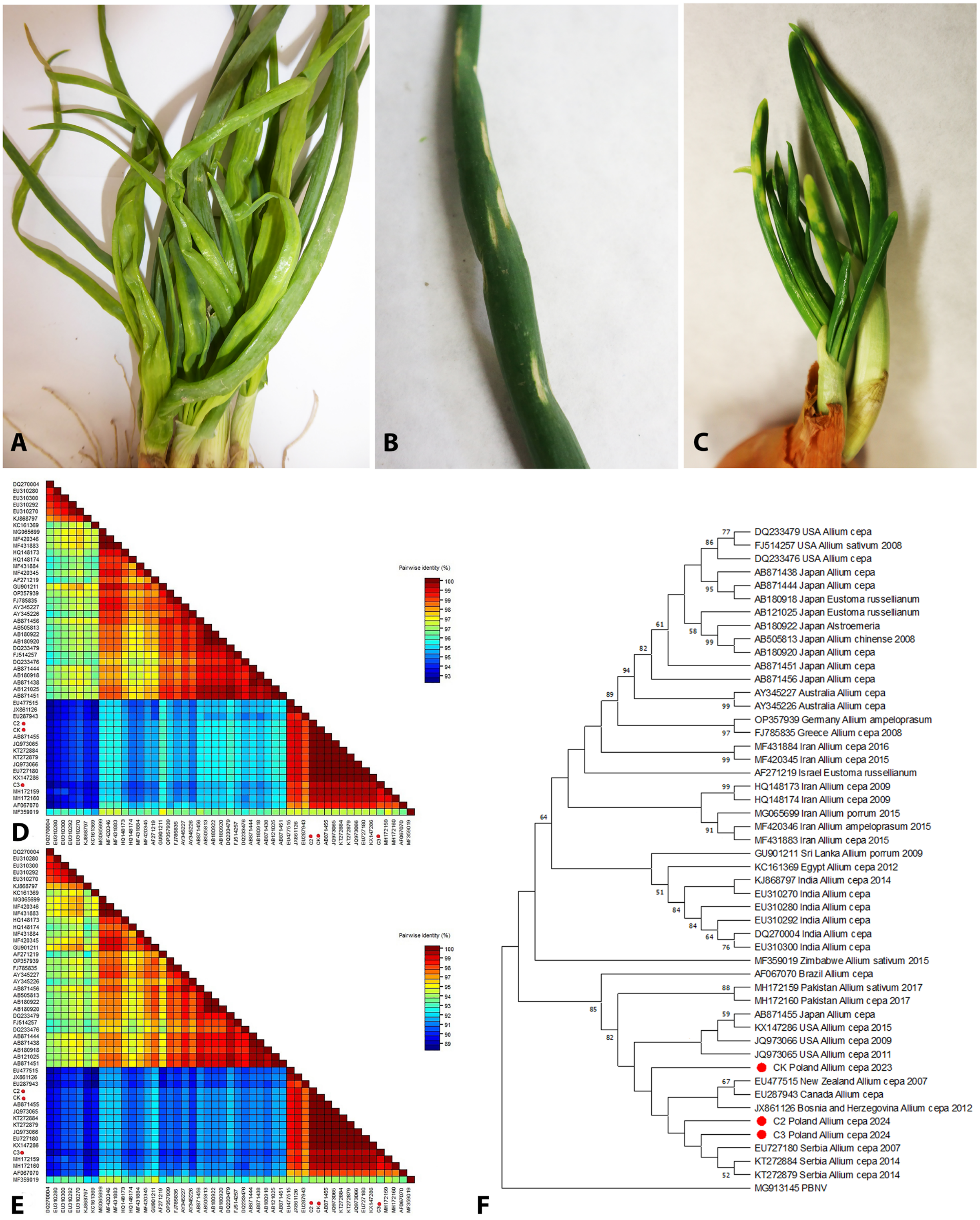
RESULTS

Detection of the viruses in collected samples

During 2023–2024, the monitoring of onion fields in Lubelskie, Wielkopolskie, and Kujawsko-Pomorskie regions of Poland was carried out. Samples were collected from symptomatic and asymptomatic onion plants.

The presence of the viruses was analysed using RT-PCR method with the specific primer pairs. OYDV was detected in five analysed samples collected from Lubelskie and Wielkopolskie regions, with the symptoms in the form of bending of leaves (Figure 1A) and yellow stripes on leaf blades. IYSV was confirmed in three samples from

Figure 1. A) Onion infected with OYDV; B) Onion infected with IYSV; C) Onion infected with IYSV and OYDV; D) Two-dimensional visualization of nucleotide and E) amino acids sequence identity of 49 IYSV isolates examined in this study. The Polish isolates are marked by red dots. The matrices were performed using SDTv1.2.; F) A phylogenetic tree of IYSV isolates created in MEGA X. The Polish isolates, obtained in this study, are marked by red dots



the same regions. These plants displayed characteristic symptoms in the form of irregular yellow-to-bleached, white-coloured changes on leaves (Figure 1B). Moreover, in one of the analysed samples, the presence of mixed infection of OYDV and IYSV was noticed. The infected plant showed symptoms of severe mosaic and leaf deformation (Figure 1C). In the remaining 32 samples, none of the tested viruses was detected.

To obtain the full-length sequence of the nucleocapsid (N) protein gene of the identified IYSV isolate, RT-PCR with newly designed primers was carried out. For the primers designed in this experiment, a temperature of 53°C was selected, as it yielded a product with the highest efficiency and a length of 1069 bp. The specificity of the obtained products was confirmed by Sanger sequencing.

As a result of sequencing, three sequences of the gene encoding the IYSV nucleocapsid (N) protein were obtained: CK 2023 (GenBank accession number PV036881) originated from Lubelskie region, C2 2024 (accession number PV036879) originated from Wielkopolskie region, and C3 2024 (accession number PV036880) originated from Wielkopolskie region.

Recombination and selective pressure analysis

The recombination analyses did not detect any recombination events in the analysed data set, allowing all sequences to be included in further phylogenetic analyses. The strength of selection acting on individual codons was evaluated using four algorithms: MEME, FUBAR, FEL and SLAC. The MEME algorithm detected the presence of positive selection ($\omega > 1$) in 8 codons (at positions 2, 85, 141, 179, 265, 266, 274, and 277), FUBAR detected the presence of positive selection in 3 codons (positions 111, 141, and 227), and FEL identified 4 codons (positions 2, 111, 141, and 264) under positive selective pressure. The SLAC algorithm did not indicate any positive selection. Negative selection ($\omega < 1$) was detected in 94 codons using the FEL algorithm, 80 codons using FUBAR, and 38 codons using SLAC.

Sequence Demarcation Tool analysis results

Distance matrix analyses of the obtained sequences with 46 full-length IYSV nucleocapsid (N) protein gene sequences retrieved from the GenBank database, performed using the Sequence Demarcation Tool, showed that nucleotide sequence identity among all analysed isolates ranged from 92% to 99%, whereas amino acid sequence identity ranged from 88% to 100% (Figure 1D, 1E). The Polish isolates obtained in this study exhibited 99% nucleotide sequence identity with each other.

Phylogenetic analysis

The phylogenetic reconstruction based on the full-length nucleocapsid (N) protein gene sequence of IYSV revealed the presence of two phylogenetic groups in the analysed population, composed of different isolates. In the first clade, isolates primarily from India, Japan, Iran, and Australia are located (Figure 1F). The Polish isolates clustered in the second clade with the isolates from Serbia, Bosnia and Herzegovina, Canada, New Zealand, the United States, Japan, Pakistan, and Brazil (Figure 1F). The only sequences that were present in both clades were those originating from Japan.

DISCUSSION

In this study, a survey of onion crops in Poland was conducted. The presence of OYDV in symptomatic plants collected in different regions of Poland (Wielkopolska and Lubelskie) was identified. OYDV infection reduces seed setting and causes premature plant death, leading to losses in onion and garlic crops ranging from 25 to 60% [Barg et al. 1994, Lot et al. 1998, Dovas et al. 2001]. The presence of OYDV in onion cultivation in Poland has been observed earlier [Taberska et al. 2021]. The virus can be transmitted through propagation materials and in a non-persistent manner by over 50 species of aphids, including *Myzus persicae*, which is the most important vector of the virus [Abd El-Wahab 2009, Jayasinghe et al. 2021]. *M. persicae* is a species present in Poland that can cause significant yield losses [Ruszkowska et al. 2017]. Climate warming promotes an increased number of aphids, which can transfer the virus from neighbouring weeds to agricultural fields, and also can adapt to new environmental conditions rather quickly. Climate change impacts vectors in several ways, including alterations in their phenology, overwintering patterns, population density, migration, and their predators. Additionally, global warming affects the primary infection of the host, the progression of the infection within the host, and/or the potential for horizontal transmission of the virus to new hosts through the vector [Harrington et al. 1995, Ruszkowska 2007].

For the first time in onion crops in Poland, the presence of another important pathogen, IYSV was also identified. IYSV was first reported in Poland in 2005 on chrysanthemum plants [Balukiewicz and Kryczyński 2005]. The virus is transmitted by onion and tobacco thrips [Srinivasan et al. 2012]. Onion thrips are common in both greenhouses and fields in Poland [Pobożniak et al. 2021]. Adult insects overwinter in the upper layer of soil, on weeds, grasses, and tobacco residues. The source of the virus can be infected seedlings and bulbs. The virus has been previously detected in other European countries (Germany, the Czech Republic), which confirms its widespread distribution in Europe.

In this study, the presence of both viruses, OYDV and IYSV, was detected in single and mixed infections. So far, mixed infections have only been observed in the case of OYDV and the viruses from the *Allexivirus* or *Potyvirus* genus, for example, *Potyvirus ampeloprasi* (leek yellow stripe virus, LYSV) [Pappu et al. 2005, Kumar et al. 2010]. Mixed infections of OYDV and LYSV not only exacerbate the health of plants and reduce yields, but can also improve the transmission efficiency of the viruses by vectors, making it more difficult to control these pathogens in *Allium* crops [Lot et al. 1998, Jayasinghe et al. 2021]. In our study, it was difficult to determine whether the presence of mixed infections may have an impact on disease symptoms. The presence of disease symptoms was observed both on samples in which OYDV or IYSV was confirmed, as well as in those samples in which the presence of viruses was not detected. This may be due to the low concentration of viruses in the tested samples. Further research is required using high-throughput sequencing, which can detect all DNA and RNA viruses present in a sample in a single assay, providing an exhaustive view of a plant's viral phytosanitary status [González-Pérez et al. 2024].

In this study, we performed the phylogenetic analysis based on the full-length sequence of the nucleocapsid (N) protein gene of IYSV. The sequences obtained in this experiment show a 99% identity, indicating low variation between isolates from two different regions of Poland. The analysis of the obtained sequences, generated through sequencing, revealed a low level of genetic diversity among samples originating from different countries. The selection pressure analysis showed that the nucleocapsid (N) protein gene has been mainly evolving under the action of purifying selection operating thus highlighting its functional role during IYSV infection. Interestingly, one codon 141 was identified as positively selected by three different methods used. This suggests that changes at this site might be beneficial to the virus and contribute to its success in infecting and replicating within the host plant.

The variability index between sequences was minimal, suggesting a high degree of genetic similarity. These findings may indicate limited genetic diversification within the studied population, regardless of the geographical origin of the samples. In the garlic-infecting isolate from Zimbabwe, the authors conducted a detailed analysis of three complete genes: nucleocapsid (N), nonstructural protein (NSs), and movement protein (NSm) [Karavina et al. 2019]. The N gene sequence did not form distinct clusters associated with specific geographical locations, suggesting a potential absence of local variants of this gene. In contrast, the NSs and NSm genes clustered closely with homologous sequences of other IYSV isolates available in databases. Their similarity to sequences from various locations may indicate the conserved functions of these genes, which are essential for the virus's infection cycle and survival across different geographical conditions [Karavina et al. 2019]. IYSV sequences were divided into three groups based on their origin. The first group consisted of sequences from North America, labeled as NL, the second group included sequences from Asian countries, labeled as BR, and the third group, named "other" exhibited the greatest genetic diversity. The sequences obtained in this study are classified in the phylogenetic tree within the NL group, which, as established in the publication, is the globally dominant [Tabassum et al. 2021].

CONCLUSIONS

In summary, the presence of IYSV in Poland has been detected for the first time in onion. Phylogenetic analysis revealed that the Polish IYSV isolates from Wielkopolskie and Lubelskie regions primarily clustered with isolates originating from Serbia. The virus is transmitted by onion thrips, a globally prevalent species, positioning IYSV as a potentially significant threat to onion cultivation both in Poland and worldwide. Another important question is regarding the occurrence of mixed infections and their impact on pathogenicity and virus transmission. There is a very limited number of complete genomic sequences of IYSV, therefore, further studies are required to elucidate the distribution, host specificity, and evolutionary dynamics of IYSV.

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REFERENCES

Abd El-Wahab, A.S. (2009). Aphid-transmission efficiency of two main viruses on garlic in Egypt, Onion yellow dwarf virus (OYDV-G) and Leek yellow stripe virus (LYSV-G). Acad. J. Entomol., 2(1), 40–42.

Bag, S., Schwartz, H.F., Cramer, C.S. et al. (2015). Iris yellow spot virus (Tospovirus: Bunyaviridae): from obscurity to research priority. Mol. Plant Pathol., 16(3), 224–237. https://doi.org/10.1111/mpp.12177

Balukiewicz, A., Kryczyński, S. (2005). Tospoviruses in chrysanthemum mother stock plants in Poland. Phytopathol. Pol. 37, 59–67.

Barg, E., Lesemann, D.-E., Vetten, H.J. et al. (1994). Identification, partial characterization, and distribution of viruses infecting allium crops in South and Southeast Asia. Acta Hortic., 358, 251–258. https://doi.org/10.17660/ActaHortic.1994.358.41

Clabbers, M.T.B., Olsthoorn, R.C.L., Gultyaev, A.P. (2014). Tospovirus ambisense genomic RNA segments use almost complete repertoire of stable tetraloops in the intergenic region. Bioinformatics, 30(13), 1800–1804. https://doi.org/10.1093/bioinformatics/btu122

Cortês, I., Livieratos, I.C., Derks, A. et al. (1998). Molecular and serological characterization of iris yellow spot virus, a new and distinct tospovirus species. Phytopathology, 88(12), 1276–1282. https://doi.org/10.1094/PHYTO.1998.88.12.1276

De Avila, A.C., Gama, M., Kitajima, E. et al. (1981). Um virus do grupo vira-cabeca do tomateiro isolado de cebola (*Allium cepa* L.). Fitopatol. Bras., 6, 525 [in Portuguese].

Derks, A.F.L.M., Lemmers, M.E.C. (1996). Detection of tospoviruses in bulbous crops and transmissibility by vegetative propagation. Acta Hortic., 432, 132–139. https://doi.org/10.17660/ActaHortic.1996.432.17

Dovas, C.I., Hatziloukas, E., Salomon, R. et al. (2001). Incidence of viruses infecting *Allium* spp. in Greece. Eur. J. Plant Pathol., 107(7), 677–684. https://doi.org/10.1023/A:1011958914573

González-Pérez, E., Chiquito-Almanza, E., Villalobos-Reyes, S. et al. (2024). Diagnosis and characterization of plant viruses using HTS to support virus management and tomato breeding. Viruses, 16(6), 888. https://doi.org/10.3390/v16060888

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Harrington, R., Bale, I.S., Tatchel, G.M. (1995). Aphids in a changing climate. In: Harrington, R., Stork, N.E. (ed.), Insects in a changing environment. Academic Press, London, 126–158.

Hasiów-Jaroszewska, B., Fares, M.A., Elena, S.F. (2014). Molecular evolution of viral multifunctional proteins: the case of potyvirus HC-Pro. J. Mol. Evol., 78(1), 75–86. https://doi.org/10.1007/s00239-013-9601-0

Huson, D.H., Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol., 2(2), 254–267. https://doi.org/10.1093/molbev/msj030

Jayasinghe, W.H., Kim, H., Sasaki, J. et al. (2021). Aphid transmissibility of onion yellow dwarf virus isolates with an N-terminal truncated HC-Pro is aided by leek yellow stripe virus. J. Gen. Plant Pathol., 87(3), 178–183. https://doi.org/10.1007/s10327-021-00986-y

Karavina, C., Ibaba, J., Gubba, A. (2019). Characterization of three full Iris yellow spot virus genes of a garlic-infecting isolate from Zimbabwe using next-generation sequencing technology. Afr. J. Biotechnol., 18, 928–934. https://doi.org/10.5897/AJB2018.16738

Kumar, S, Baranwal, V.K., Joshi, S. et al. (2010). Simultaneous detection of mixed infection of onion yellow dwarf virus and an allexivirus in RT-PCR for ensuring virus free onion bulbs. Ind. J. Virol., 21, 61–68. https://doi.org/10.1007/s13337-010-0008-x

Kumar, S., Stecher, G., Li, M. et al. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

LaTourrette, K., Garcia-Ruiz, H., (2022). Determinants of virus variation, evolution, and host adaptation. Pathogens, 11(9), 1039. https://doi.org/10.3390/pathogens11091039

Lemey, P., Salemi, M., Vandamme, A.-M. (eds.). (2009). The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. 2nd ed. Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511819049

Lot, H., Chovelon, V., Souche, S. et al. (1998). Effects of Onion yellow dwarf and Leek yellow stripe viruses on symptomatology and yield loss of three french garlic cultivars. Plant Dis., 82(12), 1381–1385. https://doi.org/10.1094/PDIS.1998.82.12.1381

Manglli, A., Mohammed, H., Hussein, A.E. et al. (2014). Molecular analysis of the 3′ terminal region of Onion yellow dwarf virus from onion in southern Italy. Phytopathol. Mediterr. 53(3), 438–450. https://doi.org/10.14601/Phytopathol_Mediterr-14027

Martin, D.P., Murrell, B., Golden, M. et al. (2015). RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol., 1(1), vev003. https://doi.org/10.1093/ve/vev003

Muhire, B.M., Varsani, A., Martin, D.P. (2014). SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PloS One, 9(9), e108277. https://doi.org/10.1371/journal.pone.0108277

Nischwitz, C., Pappu, H.R., Mullis, S.W. et al. (2007). Phylogenetic analysis of Iris yellow spot virus isolates from onion (*Allium cepa*) in Georgia (USA) and Peru. J. Phytopathol., 155(9), 531–535. https://doi.org/10.1111/j.1439-0434.2007.01272.x

Pappu, H.R., Hellier, B.C., Dugan, F.M. (2005). First report of *Onion yellow dwarf virus*, *Leek yellow stripe virus*, and *Garlic common latent virus* in garlic in Washington State. Plant Dis., 89(2), 205. https://doi.org/10.1094/PD-89-0205C

Pobożniak, M., Olczyk, M., Wójtowicz, T. (2021). Relationship between colonization by onion thrips (*Thrips tabaci* Lind.) and leaf colour measures across eight onion cultivars (*Allium cepa* L.). Agronomy, 11(5), 963. https://doi.org/10.3390/agronomy11050963

Ruszkowska, M. (2007). Across the transformation life cycle of *Rhopalosiphum padi* (L.) (Homoptera: Aphidoidea): coevolution with temperature. Rozprawy Naukowe Instytutu Ochrony Roślin 15, 60 pp.

Ruszkowska, M., Strażyński, P., Krówczyńska, A. (2017). Developmental changes of peach-potato aphid (*Myzus persicae* Sulz.) under higher temperatures – the importance of a new phenomenon in the integrated protection of potato. Progr. Plant Prot., 57(1), 16–20. https://doi.org/10.14199/ppp-2017-002 [in Polish].

Schwartz, H.F., Mohan, S.K. (2016). Introduction. In: H.F. Schwartz., S.K. Mohan (eds.), Compendium of onion and garlic diseases and pests. The American Phytopathological Society, 1–7. https://doi.org/10.1094/9780890545003.001

Shin, Y.-G., Rho, J.-Y. (2014). Development of a PCR Diagnostic System for *Iris yellow spot tospovirus* in quarantine. Plant Pathol. J., 30(4), 440. https://doi.org/10.5423/PPJ.NT.06.2014.0052

Srinivasan, R., Sundaraj, S., Pappu, H.R. et al. (2012). Transmission of Iris Yellow Spot Virus by *Frankliniella fusca* and *Thrips tabaci* (Thysanoptera: Thripidae). J. Econ. Entomol., 105(1), 40–47. https://doi.org/10.1603/EC11094

Tabassum, A., Ramesh, S.V., Zhai, Y. et al. (2021). Viruses without borders: global analysis of the population structure, haplotype distribution, and evolutionary pattern of Iris Yellow spot orthotospovirus (family *Tospoviridae*, genus *Orthotospovirus*). Front. Microbiol., 12. https://doi.org/10.3389/fmicb.2021.633710

Taberska, A., Minicka, J., Borodynko-Filas, N. et al. (2021). Identification and molecular characterization of onion yellow dwarf virus (OYDV) in *Allium cepa* crops in Poland. Progr. Plant Prot., 61(3), 214–220. https://doi.org/10.14199/ppp-2021-024 [in Polish].

Weaver, S., Shank, S.D., Spielman, S.J. et al. (2018). Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Mol. Biol. Evol., 35(3), 773–777. https://doi.org/10.1093/molbev/msx335

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RESEARCH PAPER

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RESPONSE OF ROOTING STEM CUTTINGS OF SAGE (*Salvia officinalis* L.) FOR EXOGENOUS HORMONE APPLICATIONS IN DIFFERENT GROWTH MEDIA

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ABSTRACT

The objective of this study was to investigate the effects of different growth media and IBA doses on the growth parameters of *Salvia officinalis* L. plant. The study was conducted according to a factorial experimental design in randomized plots within the climate cabin, which has fully controlled conditions, located in the Department of Horticulture at Van Yüzüncü Yıl University. A total of 360 cuttings were used, with 10 cuttings representing each application. In the study, which was conducted with three replications, plants were grown in 200 mL pots. The cuttings used in the rooting study were obtained from medicinal sage (*S. officinalis*) plants in the Medicinal and Aromatic Plants Garden of Van Yüzüncü Yıl University, Faculty of Agriculture, in June 2023. The cuttings were brought to the laboratory, planted in 3 different media: perlite (100%); perlite + peat (1 : 1); peat (100%), and applying four different concentrations of hormones: 0 ppm; 250 ppm; 500 ppm; 1000 ppm IBA (indole-3-butyric acid). The trial ended 50 days following planting, and the necessary measurements were made. In this study, the highest growth performance were obtained in the perlite + peat growth medium, except for root length. Considering the hormone × growth media interactions, the highest fresh plant weight, dry plant weight, fresh root weight, and dry root weight were obtained as 3.549 g, 0.976 g, 4.351 g, and 0.644 g, respectively, in perlite + peat growth medium applied with 1000 mg kg^{-1} IBA. The highest plant length (17.617 cm) and rooting rate (80%) were also obtained with 1000 mg kg^{-1} IBA application in peat (100%) and perlite (100%) growth media, respectively.

Key words: rooting, stem cuttings, indole-3-butyric acid, peat, perlite, substrate

INTRODUCTION

Salvia officinalis L., also known as sage, is a member of the *Lamiaceae* family and is recognized for its wide-ranging medicinal properties. Recent studies highlight the clinical efficacy of *S. officinalis* extracts. Sage leaves contain valuable bioactive compounds such as flavonoids, phenolics, rosmarinic acid, and diterpenes that contribute positively to human health and well-being [Agatonović-Kustrin et al. 2022, Aljuboori et al. 2024]. The content of bioactive compounds supports its application in various fields, including pain management, reducing inflammation, cancer treatment, cognitive enhancement, and antimicrobial action [Rodrigues et al. 2012, Erhan 2020, Aljuboori et al. 2024]. It has also been reported that it has the potential to enhance cognitive function and may play a preventive role against age-related diseases, such as Alzheimer's [Pop et al. 2016]. Furthermore, in addition to health benefits, *S. officinalis* is also recognized for its nutritional value and its use as a culinary herb, as well as a source of essential oil. Although crude essential oils may exhibit limited efficacy, their antibacterial properties

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can be enhanced through extraction, making *S. officinalis* particularly useful in food preservation and health applications [Xu 2022]. In addition, its effectiveness in managing premenstrual syndrome has been confirmed by clinical studies, indicating that it has broader implications for women's health management [Abdnezhad et al. 2019, Heydarpour et al. 2023].

Various studies have revealed the influence of growth media composition, including organic additives and basal media formulations, on the physiological traits and overall performance of plants. *Salvia officinalis* is typically cultivated using specific growth media that can significantly impact its growth, development, and essential oil production. Among the various substrates, peat and perlite mixtures have been widely studied due to their favorable physical and chemical properties. The integration of these materials affects moisture retention, aeration, and root development, all of which are important aspects to optimize for sage cultivation. Peat is consistently favored for sage germination and seedling vigor. Peat media yielding the highest germination percentages and robust seedling growth metrics for S. *officinalis*, with peat outperforming 100% soil, perlite, and sand in their trials [Aytekin et al. 2021]. This aligns with the general observation that peat-based substrates provide favorable physical properties and nutrient reservoir capacity critical for sage seedling establishment [Yoo et al. 2017, Aytekin et al. 2021]. Ferrarezi et al. [2016] determined that peat media yielded the highest germination percentage and robust seedling metrics for sage Vista Red compared with alternative substrates, reinforcing peat's suitability for *Salvia* propagation and early growth stages. Perlite is renowned for its lightweight nature and ability to improve aeration in potting mixes. Incorporating perlite into growth media can enhance aeration, but may reduce moisture retention if used in excessive amounts. The balance of perlite and other organic materials, particularly peat, is critical, with research indicating that optimal ratios significantly improve the physical and chemical properties of the medium, resulting in enhanced seedling growth [Maghdouri et al. 2021]. Care must be taken regarding excessive peat substitution with inert materials, as salinity and EC dynamics can negatively affect growth. Moderate incorporation of perlite in peat-based systems is advisable, with genotype- and system-specific validation recommended [Do and Scherer 2013]. The interaction between peat and perlite is particularly beneficial for sage propagation. Yoo et al. [2017] reported that traditional potting mixes containing combinations of soil, sand, perlite, and peat moss provided significantly better growth parameters for *Salvia* plants compared to mixes lacking these components, and peat plays a vital role in maintaining moisture, which is crucial for sage germination and early growth stages. Common peat–perlite baselines [for example, roughly 50–70% peat with 30–50% perlite] are advisable starting points, with adjustments based on irrigation and nutrient management [Ferrarezi et al. 2016, Yoo et al. 2017]. Currey et al. [2019] reported that peat and perlite mixtures play a crucial role in controlling moisture availability, which is essential for the successful cultivation of culinary herbs, including *S. officinalis*. It has been reported that the combined application of nitrogenous fertilization and peat can improve growth parameters in *S. officinalis* and optimize plant development, with the substrate playing a crucial role. Additionally, the moisture-retaining properties of peat contribute to the overall vitality of sagebrush, particularly in environments where water levels fluctuate [Sonmez and Bayram 2017]. The media having less than 25% of compost+ perlite were recommended by Do and Scherer [2013] for Salvia, underscoring the importance of substrate composition in promoting healthy growth of *S. officinalis* plants.

The application of exogenous hormones has a significant impact on physiological responses in *Salvia* species, enhancing their ability to cope with stress and promoting growth. These treatments primarily interact with the plant's endogenous hormone levels to shape responses related to stress tolerance, growth regulation, and secondary metabolite production. Application of exogenous indole-3-butyric acid (IBA), a synthetic auxin, has been extensively studied for its effects on root development and growth responses in various *Salvia* and other plant species. An optimal IBA concentration is essential, as determined in various studies involving different plant species, which have shown that too low or too high concentrations of IBA can inhibit rooting performance [Li et al. 2019]. Therefore, it has been reported that a balanced IBA application can lead to improved rooting, and consequently, increased biomass. Studies have shown that IBA application can affect secondary metabolism by increasing the stress tolerance of *Salvia* in saline conditions, thereby improving growth performance [Tan 2025]. It has been reported that IBA application can improve the rooting percentage and growth behavior of seedlings obtained from *S. officinalis* cuttings, thus effectively assisting vegetative expansion and propagation efforts [Gur et al. 2021]. In micropropagation and ex vitro rooting contexts, growth regulator regimes interacting with peat–perlite substrates influence rooting success and subsequent acclimatization. Papafotiou et al. [2023] reported that *Salvia* spp. microshoots root effectively on half-strength MS medium with low to moderate IBA (e.g., 0.0–0.5 mg L^{-1}) and acclimate successfully on peat–perlite substrates at a 1 : 1 ratio, illustrating a compatible pipeline from *in vitro* culture to peat–perlite-based *ex vitro* production. The general implication is that IBA enhances rooting in sage cuttings, and when used with peat–perlite media, rooting efficiency and acclimatization can be optimized. Although the cited study focuses on Greece-native *Salvia* species, the principle of combining IBA-treated cuttings with peat–perlite rooting substrates is broadly applicable to *Salvia* propagation [Papafotiou et al. 2023].

This study aimed to investigate the effects of different growth media and IBA doses on the growth parameters of *S. officinalis* cuttings.

MATERIALS AND METHODS

The research was conducted according to a factorial experimental design in randomized plots within the climate cabin, which has fully controlled chamber maintained at 23 °C, 65% relative humidity, with an 8/16 h dark/light photoperiod, located in the Department of Horticulture at Van Yüzüncü Yıl University. The cuttings used in the rooting study were obtained from medicinal sage (*S. officinalis*) plants from the Medicinal and Aromatic Plants Garden of Van Yüzüncü Yıl University, Faculty of Agriculture, in June 2023. Mean temperature of the month was 21.4 °C, total precipitation 7.2 mm and mean humidity was 45.4%. Cuttings of 10–12 cm length were selected from the fresh shoots of that yea, cut into cross-sections, and placed in pure water. Then, the cuttings were brought to the laboratory, and planted in 3 different media (M1 – perlite100%; M2 – perlite and peat (1 : 1); M3 – peat 100%) with applying four different levels of hormones (H0 – ethyl alcohol (99.9%) with distilled water (1 : 1) as control; H1 – 250 ppm; H2 – 500 ppm; H4 – 1000 ppm IBA. Hormone applications were done by a quick dipping method for 5 econd [Çiçek and Ozel 2021]. Some properties of materials used in growing media are given in Table 1.

Table 1. Some properties of materials used in growing media

Material	Brand	Structure	pH	Content
Peat	Klasmann TS1 Fine (white peat)	0–5 mm (extra fine)	6	Fertiliser level 1 g L^{-1} Nutrients added: 140 mg N L^{-1}, 160 mg P_2O_5 L^{-1}, 180 mg K_2O L^{-1}, 100 mg Mg L^{-1}, with necessary trace elements, Iron as EDTA chelate
Perlit	Ultraper	20–200 micron	6.8	Silicon 33.8%; Aluminium 7.2%; Potassium 3.5%; Sodium 3.4%

A total of 360 cuttings were used, with 10 cuttings representing each application. Plants were grown in 200 mL pots, and the experiment was conducted in three replications. After planting, the pots were watered with distilled water as needed. The trial ended 50 days following planting, and the necessary measurements were made. Plant height was determined by measuring the distance from the soil surface to the tip of the plant using a ruler and expressed in centimeters (cm). Plant roots were removed from the pots, and carefully cleaned by washing under water without damaging the roots. Their lengths were then determined by measuring the distance from the root collar to the tip of the root, and expressed in centimeters. Rooting rate was determined as a percentage by dividing the number of rooted cuttings by the number of planted cuttings for each application. While the fresh plant weight was determined by weighing the harvested above-ground parts immediately, the dry weight was determined after drying the plant samples in an oven at 40 °C for 24 hours. The root fresh weight was determined by weighing the roots, cleaned of soil and excess water with a napkin. The root dry weight was determined after drying the fresh root samples in an oven at 40 °C for 24 hours. The SPSS computer analysis program was used for statistical analysis of the obtained data, and the Duncan multiple comparison test was used to evaluate the differences between the means.

RESULTS AND DISCUSSION

The results of the variance analysis for plant growth parameters are given in Table 2. Plant length, fresh shoot weight, and dry shoot weight were significantly influenced ($p < 0.01$) by hormone concentration, and by the interaction between hormone level and growth medium. Growth medium alone also exerted a significant effect on plant length and fresh shoot weight ($p < 0.01$); however, no significant differences were observed in dry shoot weight among the tested media. Root length, rooting rate, and fresh root weight differed significantly ($p < 0.01$) in response to both, hormone levels and growth media. Moreover, the interaction between hormone level and growth medium significantly affected root length and fresh root weight ($p < 0.01$), as well as rooting rate ($p < 0.05$), whereas dry root weight remained statistically unchanged across all treatments (Table 2).

Table 2. The results of the analyses of variance for the plant growth parameters

Source of variation	Plant length	Fresh shoot weight	Dry shoot weight	Root length	Rooting rate	Fresh root weight	Dry root weight
Hormone (H)	109.63**	71.95**	38.73**	44.110**	13.500**	67.813 **	0.99^{ns}
Growth Media (M)	14.01**	85.48**	7.09	211.730**	67.170**	109.346**	1.05^{ns}
H × M	27.60**	28.01**	13.94**	59.440**	6.500*	23.789**	0.99^{ns}
CV	3.365	6.038	8.561	4.622	15.151	13.090	11.626

* significant at $p < 0.05$; ** significant at $p < 0.01$; ns – not significant

Mean values of plant growth parameters and the interactions between hormone concentration and growth medium are presented in Table 3. In the present study, the highest mean values for plant length, fresh shoot weight, and root length were recorded in the pure peat growth medium, reaching 15.018 cm, 2.645 g, and 15.018 cm, respectively. The greatest rooting rate, fresh root weight, and dry root weight were observed in the perlite + peat growth medium, with mean values of 66.7%, 2.569 g, and 0.344 g, respectively. In contrast, the highest dry shoot weight was recorded in perlite (0.789 g) and perlite + peat (0.762 g), which were classified within the same statistical group. Shoot growth parameters generally increased with rising hormone concentrations up to 1000 mg kg^{-1} IBA. However, the highest mean values for plant height, fresh shoot weight, and dry shoot weight were obtained at the 500 mg kg^{-1} IBA level, amounting to 15.727 cm, 2.817 g, and 0.870 g, respectively. Root growth parameters exhibited a consistent increase with increasing hormone doses, with the highest root length, rooting rate, fresh root weight, and dry root weight observed at 1000 mg kg^{-1} IBA, reaching 14.165 cm, 62.2%, 2.542 g, and 0.430 g, respectively.

Overall, the lowest values for most plant growth parameters were recorded in the perlite growth medium combined with the 0 mg kg^{-1} IBA treatment, with the exception of dry shoot weight and rooting rate (Table 3). Analysis of the hormone × growth medium interaction revealed that the highest mean values for fresh shoot weight (3.549 g), dry shoot weight (0.976 g), fresh root weight (4.351 g), and dry root weight (0.644 g) were obtained in the perlite + peat growth medium supplemented with 1000 mg kg^{-1} IBA. Furthermore, the maximum plant height (17.617 cm) and rooting rate (80.0%) were recorded in the peat and perlite growth media, respectively, when combined with the highest IBA dose.

When considering hormone dose × growth medium interactions, the lowest growth parameters were found in peat growth medium, without hormone application, except for the plant height and root length. The lowest plant height and root length were observed in perlite growth medium also without applied hormone.

In the present study, the growth media were ranked according to their ameliorative effects on plant growth parameters as follows: perlite + peat > peat > perlite. With respect to hormone treatments, IBA applications at concentrations of 500 and 1000 mg kg^{-1} exerted more pronounced positive effects on plant growth parameters compared with lower doses. Considering the interaction between hormone concentration and growth medium, the combination of 1000 mg kg^{-1} IBA with the perlite + peat substrate was identified as the most effective treatment for achieving optimal plant growth. The cultivation of *Salvia* species, particularly with regard to substrate selection, remains an important area of horticultural research. The physical and chemical properties of the growth media, including pH, drainage, and nutrient content, play a crucial role in establishing conditions favorable for root development and efficient nutrient uptake [Salamat et al. 2019]. Numerous studies have demonstrated that substrate composition, including the proportion of organic components and mineral amendments, significantly influences plant physiological responses and overall performance. In particular, the proportion of perlite in potting substrates has been shown to affect water retention and nutrient availability, both of which are essential for optimal *Salvia* growth.

Table 3. Effects of hormone dose and growth medium on growth parameters of *Salvia officinalis* L. cuttings

Parameter	Growth media	Hormone dose (mg kg^{-1})				
		0	250	500	1000	Mean
Plant length (cm)	Perlite	14.233	14.367	14.733	11.050	13.595 B
	Perlite + Peat	12.219	13.514	14.833	12.958	13.383 B
	Peat	11.400	15.500	17.617	12.833	14.337 A
	Mean	12.618 C	14.461 B	15.727 A	12.281 C	
Fresh shoot weight (g)	Perlite	1.924	1.883	2.338	1.730	1.968 B
	Perlite + Peat	1.713	2.394	2.913	3.549	2.642 A
	Peat	2.017	2.670	3.202	2.693	2.645 A
	Mean	1.884 D	2.315 C	2.817 A	2.315 B	
Dry shoot weight (g)	Perlite	0.735	0.771	0.963	0.689	0.789 A
	Perlite + Peat	0.474	0.674	0.928	0.976	0.762 A
	Peat	0.479	0.816	0.722	0.759	0.694 B
	Mean	0.562 C	0.753 B	0.870 A	0.807 B	
Root lenght (cm)	Perlite	7.600	11.783	11.392	9.850	10.157 C
	Perlite + Peat	11.403	16.764	15.644	16.258	13.725 B
	Peat	14.800	9.167	14.550	16.383	15.018 A
	Mean	11.267 C	12.572 B	13.862 A	14.165 A	
Rooting rate (%)	Perlite	40.0	53.3	80.0	80.0	63.3 A
	Perlite + Peat	66.7	66.7	60.0	73.3	66.7 A
	Peat	13.3	40.0	40.0	33.3	31.7 B
	Mean	40.000 C	53.333 B	60.000 BA	62.222 A	
Fresh root weight (g)	Perlite	0.961	1.422	1.398	1.241	1.255 C
	Perlite + Peat	1.541	1.828	2.559	4.351	2.569 A
	Peat	0.768	1.103	2.060	2.036	1.491 B
	Mean	1.089 D	1.450 C	2.005 B	2.542 A	
Dry root weight (g)	Perlite	0.144	0.192	0.210	0.224	0.192 C
	Perlite + Peat	0.237	0.217	0.280	0.644	0.344 A
	Peat	0.107	0.139	0.260	0.423	0.232 B
	Mean	0.162 C	0.182 C	0.250 B	0.430 A	

The marked with the same letters were not significantly different based on the Duncan multiple range test (DMRT); significant at $p < 0.01$

Research on culinary herbs, including *S. officinalis*, has shown that substrates composed of sphagnum peat moss combined with perlite improve volumetric water content management and promote balanced shoot and root growth through enhanced moisture regulation [Currey et al. 2019]. This is especially relevant for *Salvia* species, which are sensitive to both excessive and insufficient watering, thereby necessitating a well-aerated and well-drained substrate to ensure healthy root development. Further investigations into substrate ratios and additives have revealed that modifying peat-to-perlite proportions not only alters the physical structure of the growing medium but also influences nutrient dynamics and plant performance. Martini et al. [2022] reported that a balanced peat and perlite mixture (1 : 1, v/v) resulted in high rooting rates and successful acclimatization of *Salvia tomentosa*. Additionally, Londra [2010] highlighted the importance of hydrophobic properties arising from peat–perlite combinations, demonstrating hysteresis effects between drying and wetting cycles that affect water availability to plant roots. The favorable performance of seedlings grown in peat–perlite substrates further underscores the importance of substrate composition in supporting healthy growth of *S. officinalis*. This suggests that substrate composition not only influence early developmental stages, including germination, but also establish good conditions for strong root system formation, which is essential for successful commercial propagation.

Exogenous application of IBA has been widely studied for its role in enhancing root development and growth responses in *Salvia* species and other plants. As a synthetic auxin, IBA promotes adventitious root initiation, improves root quality, and contributes to overall plant vigor. Gur et al. [2021] demonstrated that IBA application increased rooting percentage and improved growth characteristics in *S. officinalis* cuttings, thereby supporting vegetative propagation efforts. The physiological activity of IBA is largely attributed to its conversion to indole-3-acetic acid (IAA), which regulates multiple growth processes, including root initiation [Fattorini et al. 2017]. This interaction between exogenous IBA and endogenous auxin levels highlights the importance of hormonal balance in modulating plant responses.

Previous studies have emphasized that appropriate IBA concentrations enhance rooting and biomass accumulation, whereas excessively low or high doses may inhibit rooting performance [Li et al. 2019]. IBA concentrations ranging from 600 to 2000 ppm have been reported to significantly increase root number and improve growth characteristics under various environmental conditions, including salinity stress [Sari et al. 2014, Tan 2025]. Moreover, synergistic interactions between hormonal treatments and growth media have been shown to further optimize plant growth responses, reinforcing the importance of integrating both factors in horticultural practices [Arthagama et al. 2021].

The incorporation of IBA into effective growth substrates has also been associated with enhanced physiological performance and improved antioxidant responses, facilitating plant adaptation and reinforcing the interdependence between the growth environment, hormonal regulation, and overall plant performance [Logsdon and Sauer 2016, Tan 2025]. In agreement with these findings, the present study demonstrated that the combination of perlite + peat growth medium supplemented with 1000 mg kg^{-1} IBA was the most effective treatment for promoting optimal plant growth. The results obtained are consistent with previously reported literature, confirming the suitability of combined peat–perlite substrates and appropriate IBA concentrations for enhancing growth and propagation of *Salvia* species.

CONCLUSION

The cultivation of *Salvia* species continues to attract considerable interest in horticultural research, particularly with respect to optimizing growth conditions and propagation techniques. In the present study, the highest mean values of most growth parameters were obtained in the perlite + peat growth medium, with the exception of plant height and root length. Overall, increasing IBA concentrations enhanced the growth performance of *S. officinalis* compared with treatments without hormone application. Among the tested hormone doses, 500 mg kg^{-1} IBA was more effective in promoting vegetative growth, whereas 1000 mg kg^{-1} IBA exerted a stronger influence on root development. The results further demonstrated that plant growth parameters were significantly influenced by the interaction between hormone dose and growth medium. Notably, the application of 1000 mg kg^{-1} IBA in a perlite + peat substrate proved to be the most effective treatment for achieving optimal growth of *S. officinalis*. These findings indicate that the combined use of IBA-treated cuttings and peat–perlite rooting substrates represents a practical and efficient approach for the propagation of *Salvia* species and may be broadly applicable in horticultural production systems.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Abdnezhad, R., Simbar, M., Sheikhan, Z. et al. (2019). *Salvia officinalis* reduces the severity of the premenstrual syndrome. Complement. Med. Res., 26(1), 39–46. https://doi.org/10.1159/000490104

Agatonović-Kustrin, S., Gegechkori, V., Kustrin, E. et al. (2022). The effect of lactic acid fermentation on extraction of phenolics and flavonoids from sage leaves. Appl. Sci., 12(19), 9959. https://doi.org/10.3390/app12199959

Aljuboori, I.W., Mahmood, M.S., Al-Rihaymee, S.A. et al. (2024). Clinical effectiveness of *Salvia officinalis* in periodontitis: a split-mouth randomized controlled trial. Cureus, 16(4). https://doi.org/10.7759/cureus.58582

Arthagama, I.D.M., Dana, I.M., Wiguna, P.P.K. (2021). Effect of various types of growing media and application of liquid organic fertilizer on the growth of *Dendrobium orchids*. Int. J. Biosci. Biotechnol., 8(2), 54–61. https://doi.org/10.24843/IJBB.2021.v08.i02.p07

Aytekin, R.İ., Akkamış, M., Bedir, M. et al. (2021). Effect of different growth media on germination and seedling quality of sage (*Salvia officinalis* L.) seeds. Mustafa Kemal Univ. J. Agric. Sci., 26(3), 610–616. https://doi.org/10.37908/mkutbd.956193

Çiçek, E., Ozel, A. (2021). Determination of suitable cutting type and IBA dose for seedling production in lavender (*Lavandula angustifolia* Mill.). Harran J. Agric. Food Sci., 25(2), 254–264. https://doi.org/10.29050/harranziraat.827325

Currey, C.J., Flax, N.J., Litvin, A.G., et al. (2019). Substrate volumetric water content controls growth and development of containerized culinary herbs. Agronomy, 9(11), 667.

Do, H., Scherer, C. (2013). Compost as growing media component for salt-sensitive plants. Plant Soil Environ., 59(2), 85–90. https://doi.org/10.17221/804/2012-PSE

Erhan, M.K. (2020). The potential of *Salvia officinalis* as a suppressor of cell proliferation in animal feed and human nutrition: an experimental stud. Turk. J. Vet. Anim. Sci., 44(2), 244–248.

Fattorini, L., Veloccia, A., Rovere, F.D. et al. (2017). Indole-3-butyric acid promotes adventitious rooting in *Arabidopsis thaliana* thin cell layers by conversion into indole-3-acetic acid and stimulation of anthranilate synthase activity. BMC Plant Biol., 17(1), 121. https://doi.org/10.1186/s12870-017-1071-x

Ferrarezi, R.S., Iersel, M.W.V., Testezlaf, R. (2016). Plant growth response of subirrigated salvia 'Vista Red' to increasing water levels at two substrates. Hortic. Bras., 34(2), 202–209. https://doi.org/10.1590/s0102-053620160000200009

Gur, E., Certin, M., Ozel, H.B. (2021). The effect of hormone treatments on germination and seedling characters of sage (*Salvia officinalis* L.) seeds. Alinteri J. Agric. Sci., 36(2), 62–69. https://doi.org/10.47059/alinteri/v36i2/ajas21115

Heydarpour, S., Sharifipour, F., Heydarpour, F. (2023). Effect of *Salvia officinalis* scent on postmenopausal women's sexual function and satisfaction: a randomized controlled trial. BMC Women's Health, 23(1), 442. https://doi.org/10.1186/s12905-023-02605-8

Li, Z., Ma, Y., Yin, W. et al. (2019). Exploring vegetative propagation techniques for the threatened *Pteroceltis tatarinowii* maxim using stem cuttings. HortScience, 54(4), 721–724. https://doi.org/10.21273/hortsci13729-18

Logsdon, S.D., Sauer, P.A. (2016). Nutrient leaching when compost is part of plant growth media. Compost Sci. Util., 24(4), 238–245.

Londra, P.A. (2010). Simultaneous determination of water retention curve and unsaturated hydraulic conductivity of substrates using a steady-state laboratory method. HortScience, 45(7), 1106–1112.

Martini, A.N., Vlachou, G., Papafotiou, M. (2022). Effect of explant origin and medium plant growth regulators on in vitro shoot proliferation and rooting of salvia tomentosa, a native sage of the northeastern mediterranean basin. Agronomy, 12(8), 1889. https://doi.org/10.3390/agronomy12081889

Maghdouri, M., Ghasemnezhad, M., Rabiei, B. et al. (2021). Optimizing seed germination and seedling growth in different kiwifruit genotypes. Horticulturae, 7(9), 314. https://doi.org/10.3390/horticulturae7090314

Papafotiou, M., Kargas, G., Lytra, I. (2005). Olive-mill waste compost as a growth medium component for foliage potted plants. HortScience, 40(6), 1746–1750. https://doi.org/10.21273/hortsci.40.6.1746

Pop, A.V., Tofană, M., Socaci, S.A. et al. (2016). Determination of antioxidant capacity and antimicrobial activity of selected *Salvia* species. Bull. UASVM Food Sci. Technol, 73. https://doi.org/10.15835/buasvmcn-fst:11965

Rodrigues, M.R.A., Kanazawa, L.K.S., das Neves, T.L.M. et al. (2012). Antinociceptive and anti-inflammatory potential of extract and isolated compounds from the leaves of *Salvia officinalis* in mice. J. Ethnopharmacol., 139(2), 519–526. https://doi.org/10.1016/j.jep.2011.11.042

Salamat, S.S., Hassan, M.A., Shirai, Y. et al. (2019). Application of compost in mixed media improved oil palm nursery's secondary root structure thereby reducing the fertilizer requirement for growth. J. Mol. Biol. Biotechnol., 27(3), 39–49.

Sari, F.O.S.O., Rugayah, R., Ginting, Y.C.Y.C. (2014). Pengaruh konsentrasi IBA (Indole Butyric Acid) dan jenis media tanam terhadap pertumbuhan bibit nanas (*Ananas comosus* [L.] Merr) asal tunas mahkota. J. Agrotek Tropika, 2(1), 43–48 [in Indonesian].

Sonmez, C., Bayram, E. (2017). The influence of different water and nitrogen applications on some yield parameters and antioxidant activity in sage (*Salvia officinalis* L.). Turk. J. Field Crops, 22(1), 96–103.

Tan, U. (2025). Application of indole-3-butyric acid (IBA) enhances agronomic, physiological and antioxidant traits of *Salvia fruticosa* under saline conditions: a practical approach. PeerJ, 13, e18846. https://doi.org/10.7717/peerj.18846

Xu, R. (2022). Salvia officinalis-based green-mediated vanadium nanoparticles: describing a modern chemotherapeutic drug for the treatment of colorectal carcinoma. Authorea Preprints. https://doi.org/10.22541/au.166857482.26607149/v1

Yoo, Y.K., Kim, I.K., Roh, M.S. et al. (2017). Growth, flowering, and nutrient composition of salvia grown in peat moss media containing pellets processed with poultry feather fibers at different mixing ratios. Hortic. Sci. Technol., 35(3), 289–299. https://doi.org/10.12972/kjhst.20170032

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RESEARCH PAPER

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EFFECTS OF ALGINATE ENCAPSULATED DIVALENT IONS (Zn^{2+}, Cu^{2+} AND Ca^{2+}) ON MUSTARD (*Brassica juncea* (L.) Czern.) SEED GERMINATION AND SEEDLING GROWTH

Belén Reig Vañó[1], Beata Kowalska[2], Magdalena Szczech[3], Robert Maciorowski[4], Magdalena Olkiewicz[5], Marta Giamberini[6], Bartosz Tylkowski[7]

ABSTRACT

Encapsulation of agrochemicals allows growers to precisely control the conditions under which the active ingredient is released. Since zinc, copper, and calcium ions are essential micronutrients in crop production, the goal of our work was to incorporate them into alginate-based capsules, and to investigate the impact of their release on seed germination and seedling growth. Oriental mustard (*Brassica juncea* (L.) Czern.) was selected as a model crop. Among the tested ions, Cu^{2+} exhibited the greatest increase, followed by Zn^{2+}, whereas Ca^{2+} showed the smallest increase, and its concentration declined over time when calcium-based capsules were applied. Performed studies demonstrate that released cations from the capsules into soil solution significantly affected seeds germination and biomass of mustard sprouts in laboratory tests. The release of Cu^{2+} and Zn^{2+} negatively influenced radicle development, with Cu^{2+} almost completely suppressing radicle elongation, and Zn^{2+} exhibiting a progressive inhibitory effect with increasing incubation time. Although, Ca^{2+} stimulated radicle elongation, it did not significantly affect total sprout and cotyledon biomass.

Keywords: essential micronutrients, oriental mustard, zinc, copper, calcium

INTRODUCTION

Nowadays the United Nations projects that the world's population could grow to around 8.5 billion in 2030 and 9.7 billion in 2050. It is predicted to reach a peak of around 10.4 billion people during the 2080s, and to remain at that level until 2100 [Gerland et al. 2022]. Thus, it is widely recognized that global agricultural productivity must increase to feed a rapidly growing world population. Therefore, sustainable crop production needs to be secured and enhanced.

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Encapsulation technology, protected in 1953 and commercialized in 1956 by Dr. Green and Dr. Schleicher from the USA National Cash Register Company, is considered as one of the most interdisciplinary technologies invented in the last decades [Marturano et al. 2019]. The market for encapsulated technologies keeps on growing in volume thanks to the expanding range of applications [Marturano et al. 2018, Wong et al. 2023, Woźniak-Budych et al. 2024]. Companies such as BASF, Syngenta or Ceradis have issued patent for encapsulation technologies focused on agriculture applications [Scher et al. 2003, Krieken et al. 2019, Staff et al. 2022]. The purpose of encapsulation is to enable a faster and more effective use of materials for extremely targeted delivery of active ingredients to specific places. It is achieved by protection of the load from aggressive environments and/or by controlled release within the time frame desired for the application [Bhatia 2020, Tylkowski et al. 2020, García-Carrasco et al. 2023].

Alginate, due to its outstanding properties, such as: biocompatible, biodegradable, water solubility (which minimalized the environmental problems associated with organic solvents), gelification at room temperature (which reduce the risk of the degradation of thermosensitive compounds), as well as due to its readily available and relatively inexpensive cost, has been used for seeds coating [Chin et al. 2021, 2022] as well as for agriculture capsules development [Lambrese et al. 2024]. The capsules have been especially applied to protect beneficial microorganisms or biostimulants from environmental degradation [Jíménez-Arias et al. 2023], as well as to control the release of active ingredients [Fan et al. 2022], for example, to control the release of pesticides [Du et al. 2023], herbicides [Artusio et al. 2021], or fungicides [de Castro Spadari et al. 2017]. As it is disclosed in the US Patent 5204111A [Handjani et al. 1993] alginate capsules can be produced by comprises slowly introducing an aqueous alginate solution into crosslinking solution of a bivalent metal salt. Cations employed as crosslinking agents can be arranged according to their affinity to alginate, as follows: Mn < Zn, Ni, Co < Fe < Ca < Sr < Ba < Cd < Cu < Pb [Ching et al. 2017, Reig-Vano et al. 2021]. Vinceković et al. [2017] reported encapsulation of *Trichoderma* species, as promising alternative to standard plant protection, in the matrix of alginate-based capsule crosslinked by copper cations. While Shaban and El-Komy [2001], and Qi et al. [2023] have investigated encapsulation of *Trichoderma* species in alginate capsules crosslinked with calcium cations. Furthermore, both copper and calcium ions have been used for encapsulation of plant growth regulators [Kudasova et al. 2021], or to reduce the environmental impact of copper fungicides in vineyards [Ortega et al. 2024]. Moreover, fertilizer encapsulation through alginate crosslinking with copper and zinc has been studied by Ekanayake and Godakumbura [2021]. There are a limited number of studies on the effect of alginate capsules containing Ca^{2+}, Zn^{2+} or Cu^{2+} cations on the parameters of crop seedlings. Therefore, the described research was undertaken with using *Brassica juncea* (L.) Czern. as a model plant.

The term mustard refers to a group of plants of the *Brassicaceae* (*Cruciferae*) family, which belongs to the genus *Brassica*. Mainly three species of mustard are cultivated worldwide for their gastronomic value: oriental mustard (*Brassica juncea*, used in our studies), yellow or white mustard (*Sinapis alba* L.), and black mustard (*Brassica nigra* L.). Usually, mustard plants are consumed as edible oils, condiments, sauces, fermented vegetables, or salad greens. These plants have been reported for their high nutritional value and richness in bioactive compounds such as glucosinolates, polyphenols, dietary fiber, β-carotene, and ascorbic acid [Devkota et al. 2020, Ramzan et al. 2023, Rahman et al. 2024]. Moreover, we decided to select mustard because it shows good promise as a rapid and short-duration vegetable crop which could be applied as a crop model. Indeed, it has been considered as a crop model for climate change assessment [Boomiraj et al. 2010], and as a crop model during investigation of have metals capture from contaminated soils [Nepal et al. 2024] . The aims of our work were to prepare alginate-based capsules containing Zn^{2+}, Cu^{2+} or Ca^{2+} ions in their structures and to evaluate the effect of these ions release on mustard seed germination and seedling growth. The experiments were conducted in laboratory conditions, using soil solution with released Zn^{2+}, Cu^{2+} or Ca^{2+} ions as well as soil with the alginate capsules.

MATERIALS AND METHODS

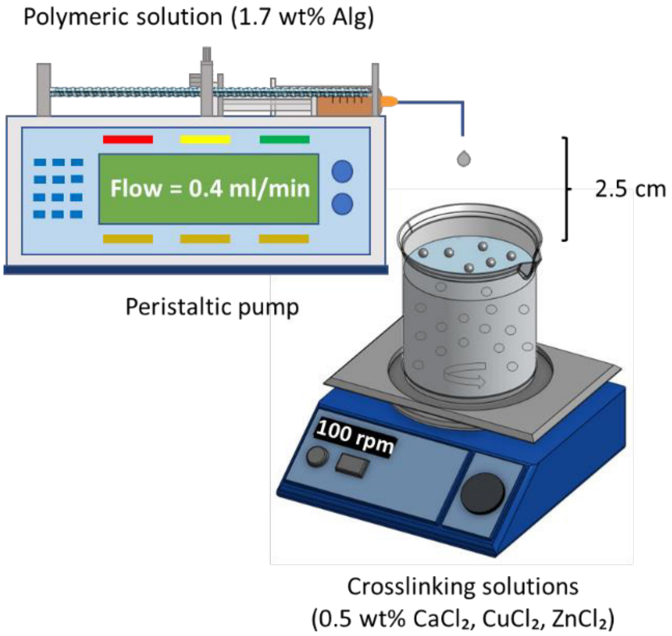
Sodium alginate was purchased from PanReac AppliChem (Barcelona, Spain). Calcium chloride anhydrous (granular, 7.0 mm, >93.0%), zinc chloride (reagent grade, >98%), braunsterican 0.5 × 16 mm BL/BL 25 G 4 5/8 needles and Norm-Ject syringes were purchased from Sigma-Aldrich (Barcelona, Spain). Copper (II) chloride dihydrate (reagent grade, >98%) was purchased from Fisher Scientific (Barcelona, Spain). Mustard *B. juncea* was provided from Kampol (Poland), and it was chosen as a test plant.

Microcapsules preparation

Three types of alginate-based microcapsules: CCa, CZn and CCu were prepared by means of a ionic gelation process during which sodium alginate was crosslinked with Ca^{2+}, Zn^{2+} or Cu^{2+} cations, respectively. As shown in

Figure 1, to fabricate the microcapsules, first 1.7 g of sodium alginate was dissolved in 98.3 g of Milli-Q water. Then, 10 g of obtained solution was extruded dropwise into 200 g of a crosslinking bath containing 0.5 wt% of aqueous solution of one of the salts: $CaCl_2$, $ZnCl_2$ or $CuCl_2$. The encapsulation process was performed at room temperature (22 ±2 °C) using a peristaltic pump (KDS 100 Legacy Syringe Pump, KD Scientific, Holliston, MA, USA) with extrusion rate set-up at 0.4 mL min^{-1}. The distance between the needle's tip and the crosslinking bath was maintained at 2.5 cm. The crosslinking solution was gently stirred, by means of a magnetic stirrer set-up at 100 rpm, during whole encapsulation process which included alginate solution extrusion, and capsules maturation periods. After 48 h of capsules maturation time [Reig-Vano et al. 2021], formed microcapsules were filtered out from the crosslinking bath, washed with Milli-Q water, and finally dried in an oven at 40 °C for 24 h.

Figure 1. Microcapsules preparation set-up



Morphology of fabricated microcapsules was observed by Leica DMS1000 Low-to-Mid Range Magnification Digital Microscope (Leica Microsistemas S.L.U., Barcelona, Spain) and by Environmental Scanning Electron Microscopy (ESEM) using a FEI ESEM Quanta 600 (FEI, Eindhoven, Netherlands) in low vacuum at an intensity of 15 kV.

Microcapsules effect on mustard germination

To investigate the effect of the encapsulated cations on the mustard germination, the mustard seeds were treated with one of 10 different types of soil solutions: SS-0; SS-CCa-3d; SS-CCa-6d; SS-CCa-9d; SS-CZn-3d; SS-CZn-6d; SS-CZn-9d; SS-CCu-3d; SS-CCu-6d; or SS-CCu-9d. In the acronyms:

"SS" means soil solution. It was prepared by thoroughly mixing of 6 kg of the soil with 6 L of water and its incubation for 24 h at ambient temperature. The solution was filtered through filter paper to remove soil particles. SS-0 refers as a control. The soil used in the experiments was sandy-loam soil collected in the National Institute of Horticultural Research (Skierniewice, Poland) and contains: N-NO_3 – 53 mg; P – 64 mg; K – 107 mg; Mg – 131 mg and Ca – 705 mg per 1 L of soil.

"CCa", "CZn" and "CCu" refer to the type of microcapsules, which were used for $Ca^{2+,}$ Zn^{2+} and Cu^{2+} cations release from the CCa, CZn, CCu microcapsules, respectively. These releases were carried out by mixing 50 mL of SS-0 with 0.5 g of corresponding microcapsules and incubated at 25 °C.

"3d", "6d", "9d" corresponds to number of days during which the SS-0 was incubated with the microcapsules. As a matter of examples "SS-CCu-9d" means that this solution was obtained by mixing 0.5 g of CCu microcapsules with 50 mL of SS-0, and then incubated for 9 days. After this period the soil solution was used in the mustard germination test. Each soil solutions were prepared in triplicate.

The tests were performed in Petri plates, lined with disks of filter paper soaked with soil solution added with the capsules. The soil solution "SS" was prepared as described above. Then solution was divided into 50 ml falcon tubes (50 ml of the solution per tube) and was added with 0.5 g of the capsules containing Ca, Zn or Cu cations. Soil solution without capsules (SS-0) served as a control. Each treatment was made in triplicates. The tubes were incubated in dark for 3; 6 and 9 days at 25 °C. After 3 days of incubation, and then after 6 and 9 days, the soil solution with capsules in falcon tubes was vortexed and 2 ml was poured into Petri plate (90 mm diam.) lined with sterile cellulose filter paper. Six plates were prepared for each type of the soil/capsule solution, for each time of incubation. Fifteen seeds of *B. juncea* were placed on the saturated filter paper in each plate. The plates with the seeds were incubated in the growth chamber (Sanyo MRL-351H, Japan) for 14 h in the light at 20 °C, and 10 h in dark at 18 °C, for 10 days. After incubation, mustard sprouts were counted to evaluate germination. Then the sprouts were extracted from the plates to measure: radicle length and weight, shoot weight, and total weight. The test was repeated once at the same conditions.

The effect of the capsules added to soil on mustard seedling growth

The same soil, as for preparation of the soil solution for germination tests, was used in the experiment on the effect of cations release from the capsules on the growth of mustard seedlings. For this experiment the soil was mixed with perlite 3–6 mm (Biovita, Poland) at the volume ratio 3 : 1, and distributed into plastic containers (11 × 17 × 5 cm), 600 g of the soil mixture per one container. The capsules (6 g; 1%) were added to the soil in each container, and thoroughly mixed. The soil without capsules was prepared as a control. Then, water was added to the soil in each container, to obtain approx. 60% of moisture. Closed containers were incubated at ambient temperature for 7 and 14 days.

The next step of this experiment was performed with the use of Phytotoxkit (Tigret, Poland), which is intended for phytotoxicity screening of chemicals, leachates in soil or in other solid, bulk materials. The evaluation is carried out in special, transparent, plastic plates (21 × 15.5 × 0.5 cm), divided to two chambers, one of which is filled with soil sown with test plant seeds. Construction of the plates allows direct observation and measurement of the length of the testing plants.

After one week of incubation, 180 g of the soil with the capsules was taken from each container and distributed into three Phytotoxkit plates (60 g of soil per one plate). Ten seeds of *B. juncea* were then sown into each plate. The plates were incubated in the growth chamber (14 h in the light at 20°C, and 10 h in dark at 18°C). The same was repeated after 14 days of soil incubation in the containers. The plates were incubated for 10 days. Thereafter, mustard seedlings were extracted carefully and following parameters were measured: radicle length and weight, shoot weight, total plant weight. The tests were repeated once at the same conditions.

Chemical analysis

The soil solutions incubated with studied capsules, as well as the bulk soil added with these capsules, were subjected to chemical analysis. For soil solutions, three samples (20 ml) of each solution were taken after 3, 6 and 9 days of incubation and analyzed for the content of available forms of calcium, zinc and copper. For bulk soil mixed with the capsules, the samples (50 g) were taken after 7, and then after 14 days of incubation, and studied for available forms of elements mentioned above. The samples for each treatment were analyzed in triplicates. Determination of Ca, Zn and Cu in soil solution was directly performed using an ICP-OES plasma spectrometer model Optima 2000 DV (Perkin-Elmer), with wavelengths: 327.393 nm for Cu; 317.933 nm for Ca, and 206.200 nm for Zn. This method is widely applied in the network of Chemical and Agricultural Stations in Poland for diagnostic purposes. To measure calcium content, the soil was extracted with acetic acid solution (0.03 N), at a volume ratio of soil : acetic acid solution 20 g : 200 ml, and mixed on a rotary stirrer for 30 min. After filtration the calcium concentration was measured by ICP at the wavelength described above. For copper and zinc, the soil was extracted with Lindsey solution based on EDTA and citric acid at a ratio of vol. 25 : 100 ml. After mixing on a rotary stirrer and filtration, these elements contents were determined with ICP at mentioned wavelengths.

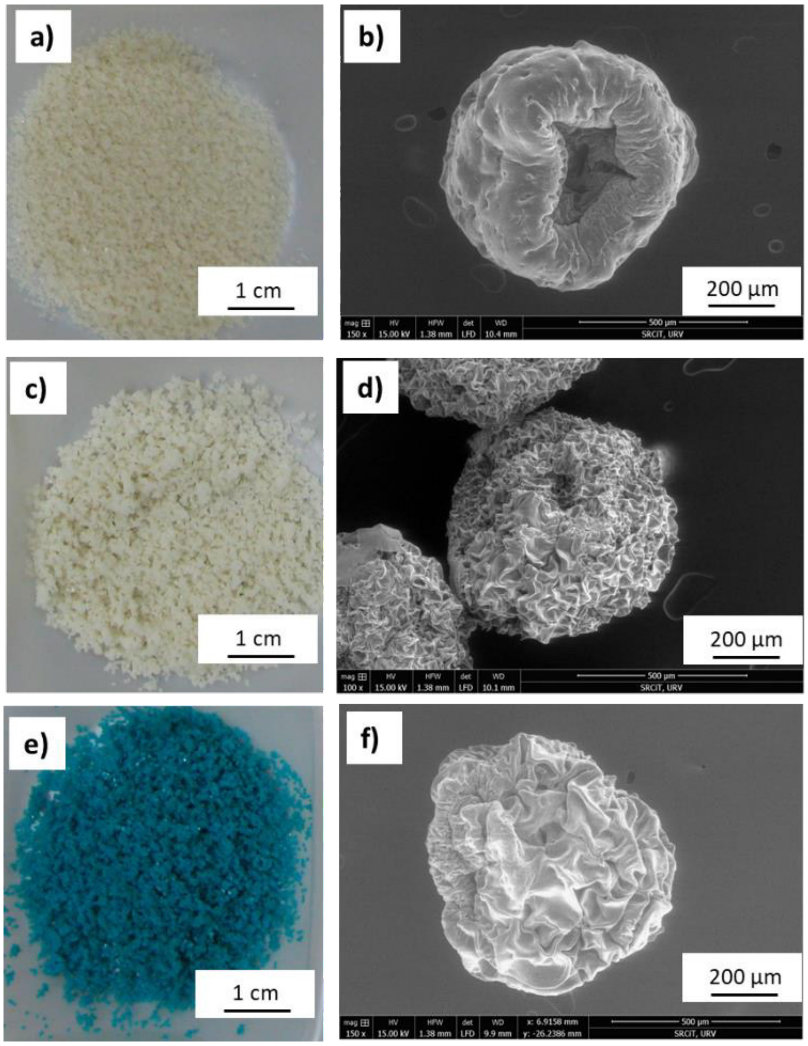
Statistical analyses

Data from the experiments were tested for normality of distribution and homogeneity of variance using Shapiro-Wilk and Lavene tests, respectively. An analysis of variance was then performed in a two-factor model with the variance due to the repetition of experiments extracted. If significance was found for the effects tested, the means were compared using Duncan's test at $\alpha = 0.05$. Calculations were performed in the statistical package Statistica v. 13 (Dell Inc. 2016).

RESULTS AND DISCUSSION

By applying the external ionotropic gelation method, three types of microcapsules formed by alginate, and calcium, zinc or copper cations were successfully fabricated. Figure 2a, c, and e shows the digital images of CCa, CZn and CCu microcapsules, respectively, while Figure 2b, d and f shows their ESEM micrographs.

Figure 2. Digital images (a, c and e) and ESEM micrographs (b, d and f) of CCa (a, b), CZn (c, d) and CCu (e, f) microcapsules



From the digital micrographs it seems that the microcapsules are aggregated, and several of them fuse together to form clumps, which could affect their stability and eventually leads to caking of particles. However, their deeper investigation by ESEM demonstrate that they are well separated. The difference in their outer morphology is remarkable. The surface of the calcium-crosslinked microcapsules (Figure 2b) is smooth and uniform, while the surfaces of the capsules crosslinked by zinc and copper cations (Figure 2d and 1f, respectively) are extremely rough and irregular. Reig-Vano et al. [2021] reported that this difference can be attributed to a different crosslinking mechanism.

Kinetics release of cations from the capsules structure into soil solution and bulk soil

In all experiments with mustard, concentrations of the cations released from the capsules were studied, according to the time of their incubation in soil solution or in bulk soil. The content of available calcium, zinc and copper ions did not change in the soil solution as well as in the soil during whole time of incubation (Tables 1 and 2).

When the capsules with Ca, Zn or Cu were added to the soil solution (after 3 days of incubation), the concentration of the respective cations significantly increased by 53%, 926%, and 232%, respectively (Table 1). After next 3 days of incubation, no further increase in concentrations of these cations in the amended solutions was noted. However, significant increase of studied cations concentrations was recorded after 9 days. It was also observed that application of capsules with Zn^{2+} also resulted in growth of the concentration of available Cu^{2+}, and opposite, application of Cu-capsules caused increased amount of available Zn^{2+} (Table 1).

McBride and Bouldin [1984] carried out studies based on ion-selective electrode data, and they reported that at least 99.5% of Cu in soil was in an organically complexed form, while Hazra et al. [1987] reported that more than 84% of total Zn in soils occurs as structurally lattice bound, about 13% as sesquioxide bound, 1.6% as organically complexed, and approximately 1% as exchangeable and water soluble forms. According to literature [Shaheen et al. 2013, Yu et al. 2023] pH value of the soil system is a very important parameter, directly influencing sorption/desorption, precipitation/dissolution, complex formation, cation exchange capacity and oxidation-reduction reactions. In general, maximum retention of cationic metals by soil active sides occurs at pH > 7. Indeed, Gong and Donahoe [1997] reported that Cu and Zn became mobile from the soil to soil solution by decreasing its pH value. Thus, very low concentrations of Cu and Zn cations detected in SS0 soil solution (0.02 and 0.01 mg L^{-1}, respectively) could be explained by its pH value of 7.7. At this pH values Cu and Zn cations can be retented by soil active sides. While, in soils solutions enriched with zinc and copper capsules (SS-CZn and SS-CCu) pH values decreased from pH 7.7 to 7.1 and 6.3 respectively. It seems that these pH value changes caused Cu and Zn cations released from the soil, and as consequence they were detected in the investigated soil solutions. Similar increase of soluble Cu^{2+} and Zn^{2+} concentration in soil solution was noted after application of Ca-capsules (soil solution pH 7.2). However, addition of Zn- and Cu-capsules to the soil solutions reduced availability of Ca presented in the stock solution, ~32% and ~36%, respectively, after 9 days of incubation. In the case of Zn, the reduction of Ca availability was significant during the first 3 days of incubation. This phenomena could be explained by Ca and Zn ions exchangeability.

Table 1. Concentration of soluble elements in soil solutions incubated for 3, 6 and 9 days

Treatment	Ca (mg L^{-1})			Zn (mg L^{-1})			Cu (mg L^{-1})		
	Days of soil solutions incubation								
	3	6	9	3	6	9	3	6	9
SS0	110.7 c	112.0 c	113.0 c	0.01 c	0.01 c	0.01 c	0.02 c	0.01 c	0.01 c
SS-CCa	168.3 b	170.0 b	200.7 a	0.36 c	0.06 c	0.37 c	0.20 c	0.02 c	0.16 c
SS-CZn	90.7 e	88.5 e	77.2 f	92.60 b	94.80 b	123.20 a	0.84 c	0.80 c	0.66 c
SS-CCu	106.7 d	104.0 d	71.6 g	0.08 c	0.07 c	0.08 c	46.50b	50.00b	73.20 a

SS0 – incubated stock soil solution (control); SS-CCa, SS-CZn, SS-CCu soil solutions incubated with microcapsules of Ca, Zn, and Cu, respectively. Means followed by the same letter do not differ significantly according to DMRT at p ≥ 0.05

Table 2. Concentration of elements available for plants in soil incubated for 7 and 14 days with the capsules

Treatment	Ca (mg kg^{-1})		Zn (mg kg^{-1})		Cu (mg kg^{-1})	
	Days of incubation in soil					
	7	14	7	14	7	14
S0	837.3 c	768.0 d	11.6 c	10.5 c	2.2 c	3.0 c
S-CCa	1006.7 a	877.3 b	8.7 c	9.5 c	1.5 c	2.0 c
S-CZn	828.0 c	705.7 e	1369.0 b	1501.7 a	2.1 c	1.9 c
S-CCu	823.0 c	760.7 d	9.2 c	9.8 c	914.9 b	977.4 a

SS0 – incubated stock soil solution (control); SS-CCa, SS-CZn, SS-CCu soil solutions incubated with microcapsules of Ca, Zn, and Cu, respectively. Means followed by the same letter do not differ significantly according to DMRT at p ≥ 0.05

In bulk soil, just like in the soil suspension, incubation time was not the factor changing the concentration of available cations. Addition of the capsules to the soil mixture with perlite resulted in significant growth of soluble cations amount compared to control soil (S0) not supplemented with the capsules (Table 2). For copper, after

7 days of soil incubation, the concentration of this element increased more than 400-times compared to control, and successively increased after 14 days of incubation. To the lower extend increased concentration of soluble zinc (about 100-times after 7 days), but also in the case of this ion, progressed availability was observed with time of incubation. On the contrary, calcium concentration increase, although significant after the addition of capsules, was lower compared to Cu and Zn. Moreover, in the case of calcium added with the capsules, its availability decreased with time of incubation, and was significantly lower after 14 days than after 7 days.

Effect of encapsulated cations on mustard seeds germination and sprouts biomass

Cu, Zn and Ca, released from the capsules into soil solution significantly affected seeds germination and biomass of mustard sprouts in laboratory tests. Seed germination was the most influenced by copper, especially after treatment with the nine-day-old incubated soil solution (Figure 3a), where mustard germination was inhibited by about 50% compared to control. Similar effects were observed by Rather et al. [2020] who exposed *B. juncea* to 3.0 mM copper aqueous solution for 3 days, as well as by Gautam et al. [2016] who investigated the changes in the safflower (*Carthamus tinctorius* L.) growth during its exposure to different concentrations of copper (25, 50 and 100 µM) for 20 days. In our studies we also observed that in excess of its permissible concentration, copper cations can lead to a significant reduction of seed germination and seedling growth, while zinc and calcium did not affect mustard germination significantly. Similar effect was observed in order to total sprouts weight, which was significantly reduced by copper released form the CCu capsules (Figure 3c). Zhao et al. [2022] and Xin et al. [2020] also indicated, that high concentration of copper can have negative impact on seeds germination and plant growth. While, in the studies of Bączek-Kwinta et al. [2020] it was found that zinc unaffected germination of broccoli (*Brassica oleracea* L. var. *botrytis italica*), sunflower (*Helianthus annuus* L.), and pea (*Pisum sativum* L.) seeds.

In our studies, total biomass of the sprouts was not affected by zinc and calcium released from CZn and CCa capsules. However, the cations had different effects on the ratio of radicle to cotyledons weight. The strongest effect of the cations released to soil solution was observed in the case of sprout's radicles. Copper and zinc released from CCu and CZn capsules, greatly reduced mustard radicles, especially their length (Figure 3c and d, respectively). In the case of copper, radicle development was almost completely reduced regardless of the incubation time of soil solution with the CCu capsules. Zinc also reduced the radicle development, but its effect was enhanced proportionally with increased incubation time of CZn capsule-soil solution by: 38%, 70%, 94% for 3, 6 and 9 days of capsules incubation, respectively. The results are consistent with those obtained by Ivanova et al. [2010] and Wen et al. [2024] who shown that copper and zinc have influenced on the parameters of plants.

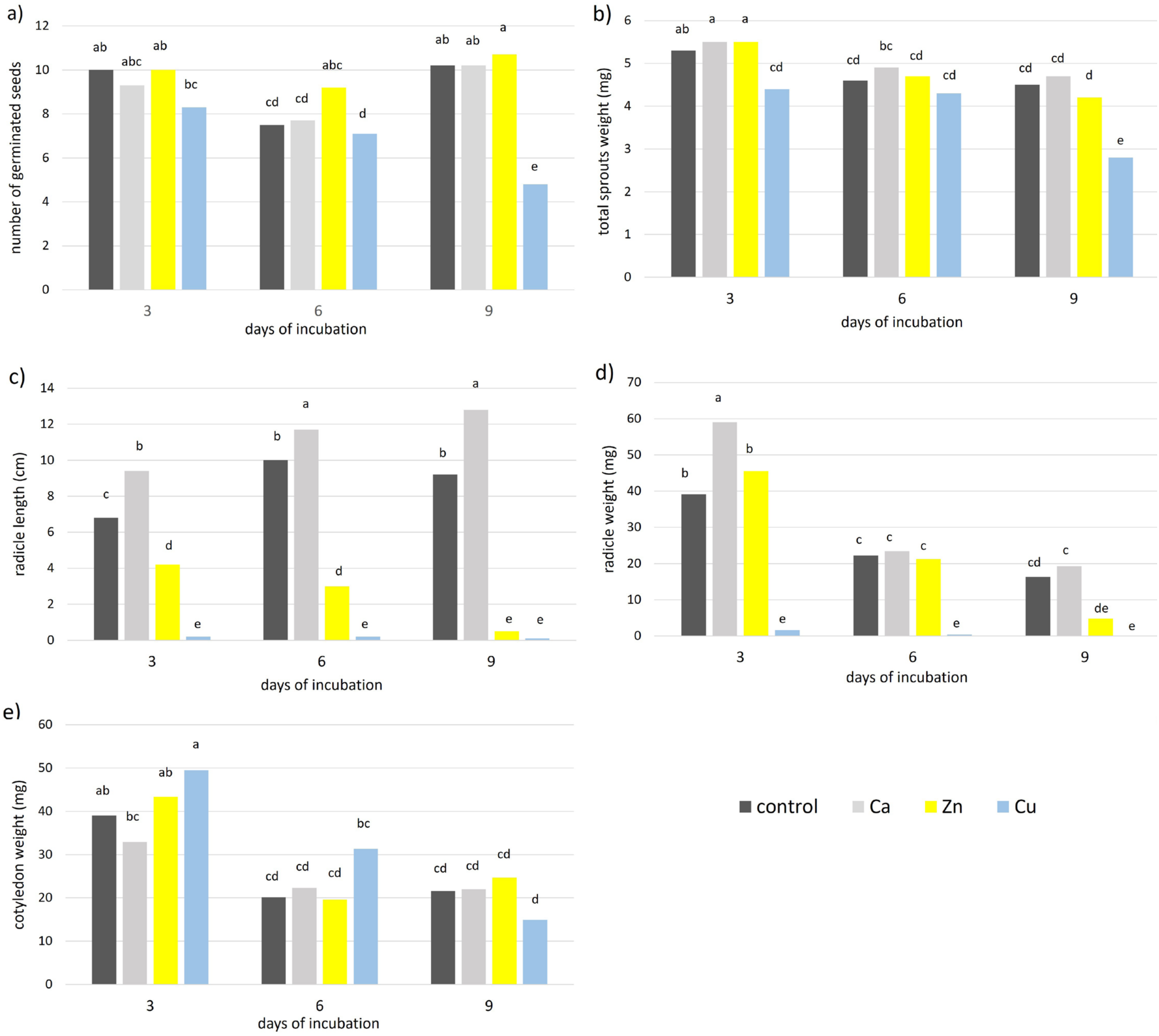
In opposite, calcium significantly stimulated sprouts radicle elongation (Figure 3c), and its effect was positively proportional to the time of its release from the CCa capsules. When the soil solution incubated with the CCa capsules for 9 days was used, the mustard radicles were 39% longer compared to control sprouts. Actually, Li et al. [2014] reported that the treatment of wheat seeds (*Triticum aestivum* L.) with aqueous solution of zinc sulfate heptahydrate $ZnSO_4·7H_2O$ (concentration range from 0.5 mM to 3 mM) did not affect seed germination, but reduced root length, while Takahashi et al. [1992] reported that Ca^{2+} at 10 or 20 mM, significantly stimulated root elongation in *P. sativum* and *Zea mays* L. seedlings.

A different response to the ions applied with capsules was found on cotyledons. In this case, copper released to the soil solution stimulated wight of this part for 27% and 55% after 3 and 6 days of incubation, respectively, compared to control (Figure 3e). However, the differences were not significant. After 9 days of incubation in soil solution, concentration of copper released from the capsules was phytotoxic. Zinc and calcium, released from the CZn and CCa capsules, had no significant effect on cotyledons biomass.

The effect of capsules added to soil on mustard seedling growth

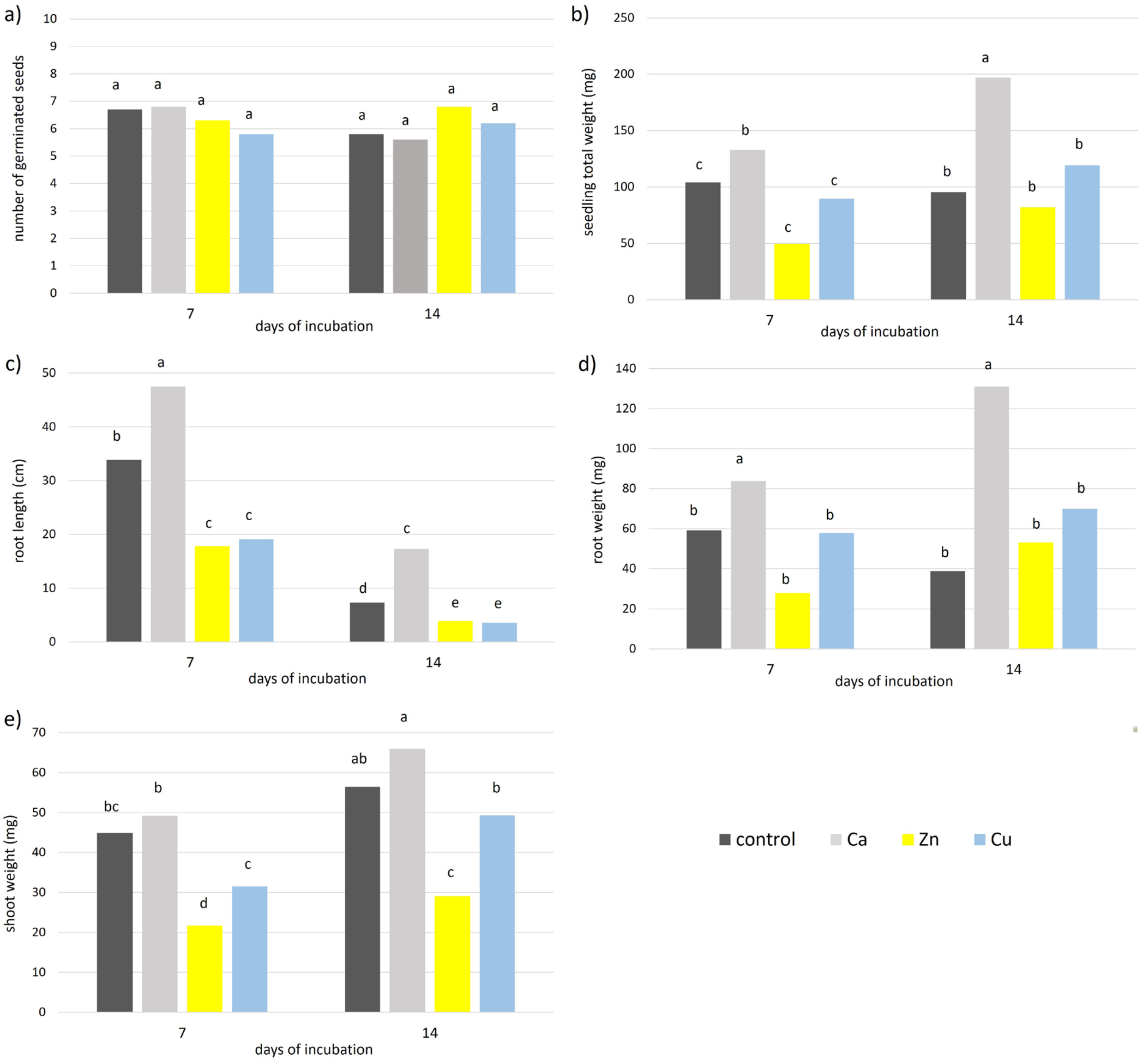
The cations released from the capsules to the soil had not significant effect on mustard seeds germination in Phytotoxkit tests. The germination was comparable for all treatments in both incubation times (Figure 4a). However, the seedling weight was significantly enhanced by calcium released to the soil from the capsules (Figure 4b). Stronger, positive effect of calcium on mustard growth was observed in soil incubated for 14 days, where the seedlings from soil with Ca-capsules were by 106% bigger than in control. This positive effect of calcium was mostly related to the greater roots development, both length and weight (Figure 4c and 4d). The average length of the roots in soil amended with Ca-capsules was 29% and 58% higher than in control for plants grown in soil incubated for 7 and 14 days, respectively. Similarly, the weight of mustard roots treated with released Ca was 29% and 70% greater than for control plants grown in soil incubated for 7 and 14 days, respectively. Moreover, calcium released from the capsules stimulated also the growth of the above-ground parts of mustard seedlings (Figure 4e), however, the differences were not significant.

Figure 3. The effect of the ions released from capsules into soil solution on mustard sprouts development: germination (a); total weight (b); radicle length (c); radicle weight (d); cotyledon weight (e). The same letters above the bars indicate that means are not significantly differentiated according to DMRT at p ≥ 0.05



In the case of copper and zinc, these elements released to the soil during incubation, significantly decreased roots elongation (Figure 4c). However, these cations did not affect significantly the weight of the young roots (Figure 4d). In those experiments, zinc also significantly inhibited development of the above-ground parts of the seedlings (Figure 4e), what resulted in decreased total seedlings weight (Figure 4b). Copper had no marked influence on mustard seedling growth, regardless the duration of the capsules incubation in the soil. Mariano-da-Silva et al. [2025] investigated the effects of different copper concentrations on the germination and seedling development of canola (*Brassica napus* L. var. *oleifera*). Canola is an oilseed crop developed through genetic improvement of rapeseed, which originated from a spontaneous interspecific cross between wild cabbage (*Brassica oleracea* L.) and mustard (*Brassica rapa* L. syn. *campestris*). The authors reported that copper levels exceeding approximately 30–90 mg Cu kg^{-1} soil, depending on species and soil conditions, inhibit *Brassica* growth, reducing chlorophyll content, photosynthetic efficiency, nitrate reductase activity, and water relations, while increasing oxidative stress markers. Moreover they demonstrated that in case of *B. juncea*, exposures to 30–90 mg Cu kg^{-1} resulted in pronounced reductions in growth parameters and key biochemical processes. Similarly, *Brassica napus* L. seedlings exposed to 200–800 mg L^{-1} Cu exhibited significant declines in germination, root and shoot elongation,

Figure 4. The effect of the ions released from capsules on mustard seedling growth in treated soil: seeds germination (a); total seedlings weight (b); root length (c); root weight (d); shoot weight (e). The same letters above the bars indicate that means are not significantly differentiated according to DMRT at p ≥ 0.05



and dry mass accumulation. Long et al. [2003] reported that zinc toxicity thresholds in *Brassica* typically range from 170–270 mg DTPA-Zn kg^{-1} soil, levels above which biomass and yield begin to decline. In *Brassica chinensis* L. (pakchoi, Chinese cabbage), shoot biomass dropped when soil-extractable Zn exceeded ~170 mg kg^{-1}, and phytotoxic effects in edible tissues were observed at total soil Zn concentrations of 224–413 mg kg^{-1}. The authors indicated that across heavier soils, other *Brassica* vegetables showed similar declines in growth and photosynthesis under elevated Zn.

CONCLUSIONS

The incubation time of alginate-based capsules in soil did not significantly affect the concentration of available cations. Among the tested ions, Cu^{2+} exhibited the highest increase, followed by Zn^{2+}, whereas Ca^{2+} concentration not only displayed the lowest increase, but also declined over time when calcium-based capsules were applied. The release of Cu^{2+} exerted the strongest impact on plant performance, significantly inhibiting seed germination and reducing sprout biomass. In contrast, Ca^{2+} and Zn^{2+} did not significantly affect mustard germination nor total biomass of the sprouts. The release of Cu^{2+} and Zn^{2+} from the capsules hindered radicle development. Cu^{2+} almost completely inhibited radicle development, while Zn^{2+} had a progressive inhibitory effect with increased incubation time. In contrast, Ca^{2+} stimulated radicle elongation. Although Cu^{2+} increased cotyledon weight, prolonged incubation (9 days) led to phytotoxic effects associated with elevated Cu^{2+} concentrations. Neither Ca^{2+} nor Zn^{2+} significantly affected cotyledon biomass.

The findings of this study suggest that alginate-based encapsulated ions have potential applications in controlled nutrient delivery; however, ion-specific responses and phytotoxic thresholds must be carefully considered. These results justify further evaluation using economically important vegetable crops. Future research should focus on the development of alginate-based capsules crosslinked with zinc, copper, and calcium, with diameters smaller than 1 mm, to assess how capsule size influences cation release dynamics in soil and subsequent plant growth responses.

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AUTHOR CONTRIBUTIONS

M.O., B.T., M.G., M.S., B.K. – methodology, B.T., M.S., M.G. – conceptualization; B.T., M.G., M.O., R.M. – data curation; B.R. – formal analysis; B.R., B.K., M.S. – investigation; M.O., B.T., M.G., M.S., B.K – methodology; B.T. – project administration; B.T., M.G., B.K., M.S. – resources; B.T., M.G. – supervision; B.T., M.S. – visualization; B.R., B.T., M.G., M.O., M.S. – writing – original draft preparation; B.T., B.K., M.S. – writing – review and editing. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors ensure that they have neither professional nor financial connections related to the manuscript sent to the Editorial Board. The funders 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.

REFERENCES

Artusio, F., Casà, D., Granetto, M. et al. (2021). Alginate nanohydrogels as a biocompatible platform for the controlled release of a hydrophilic herbicide. Processes, 9, 1641. https://doi.org/10.3390/pr9091641

Bączek-Kwinta, R., Baran, A., Simlat, M. et al. (2020). Enrichment of different plant seeds with zinc and assessment of health risk of Zn-fortified sprouts consumption. Agronomy, 10, 937. https://doi.org/10.3390/agronomy10070937

Bhatia, M. (2020). A review on application of encapsulation in agricultural processes. In: S.H., Sonawane, B.A., Bhanvase, M., Sivakumar (eds.), Encapsulation of active molecules and their delivery system. Elsevier, 131–140. https://doi.org/10.1016/B978-0-12-819363-1.00008-9

Boomiraj, K., Chakrabarti, B., Aggarwal, P.K. et al. (2010). Assessing the vulnerability of Indian mustard to climate change. Agric. Ecosyst. Environ., 138, 265–273. https://doi.org/10.1016/j.agee.2010.05.010

de Castro Spadari, C., Lopes, L.B., Ishida, K., (2017). Potential use of alginate-based carriers as antifungal delivery system. Front. Microbiol., 8. https://doi.org/10.3389/fmicb.2017.00097

Chin, J.M., Lim, Y.Y., Ting, A.S.Y. (2021). Biopolymers for biopriming of *Brassica rapa* seeds: a study on coating efficacy, bioagent viability and seed germination. J. Saudi Soc. Agric. Sci., 20, 198–207. https://doi.org/10.1016/j.jssas.2021.01.006

Chin, J.M., Lim, Y.Y., Ting, A.S.Y. (2022). Biopriming *Pseudomonas fluorescens* to vegetable seeds with biopolymers to promote coating efficacy, seed germination and disease suppression. J. Saudi Soc. Agric. Sci., 21, 493–505. https://doi.org/10.1016/j.jssas.2022.02.002

Ching, S.H., Bansal, N., Bhandari, B. (2017). Alginate gel particles – a review of production techniques and physical properties. Crit. Rev. Food Sci. Nutr., 57, 1133–1152. https://doi.org/10.1080/10408398.2014.965773

Devkota, K., dos Santos, C.F., Blochtein, B. (2020). Mustard plants distant from forest fragments receive a lower diversity of flower-visiting insects. Basic Appl. Ecol., 47, 35–43. https://doi.org/10.1016/j.baae.2020.05.005

Du, Y., Zhang, Q., Yu, M. et al. (2023). Sodium alginate-based composite microspheres for controlled release of pesticides and reduction of adverse effects of copper in agricultural soils. Chemosphere, 313, 137539. https://doi.org/10.1016/j.chemosphere.2022.137539

Ekanayake, S.A., Godakumbura, P.I. (2021). Synthesis of a dual-functional nanofertilizer by embedding ZnO and CuO nanoparticles on an alginate-based hydrogel. ACS Omega, 6, 26262–26272. https://doi.org/10.1021/acsomega.1c03271

Fan, C., Liu, Y., Dang, M. et al. (2022). Polysaccharides synergistic boosting drug loading for reduction pesticide dosage and improve its efficiency. Carbohydr. Polym., 297, 120041. https://doi.org/10.1016/j.carbpol.2022.120041

García-Carrasco, M., Valdez-Baro, O., Cabanillas-Bojórquez, L.A. et al. (2023). Potential agricultural uses of micro/nano encapsulated chitosan: a review. Macromol, 3, 614–635. https://doi.org/10.3390/macromol3030034

Gautam, S., Anjani, K., Srivastava, N. (2016). In vitro evaluation of excess copper affecting seedlings and their biochemical characteristics in *Carthamus tinctorius* L. (variety PBNS-12). Physiol. Mol. Biol. Plants., 22, 121–129. https://doi.org/10.1007/s12298-016-0339-1

Gerland, P., Hertog, S., Wheldon, M. et al. (2022). World Population Prospects 2022: Summary of results. United Nations, New York.

Gong, C., Donahoe, R.J. (1997). An experimental study of heavy metal attenuation and mobility in sandy loam soils. Appl. Geochem., 12, 243–254. https://doi.org/10.1016/S0883-2927(96)00049-2

Handjani, R.-M., Kauffmann, M., Huguenin, F. (1993). Process for the preparation of alginate capsules, apparatus for producing said capsules and cosmetic compositions containing said capsules. United States Patent, US5204111A.

Hazra, G.C., Mandal, B., Mandal, L.N. (1987). Distribution of zinc fractions and their transformation in submerged rice soils. Plant Soil., 104, 175–181. https://doi.org/10.1007/BF02372530

Ivanova, E.M., Kholodova, V.P., Kuznetsov, V.V. (2010). Biological effects of high copper and zinc concentrations and their interaction in rapeseed plants. Russ. J. Plant Pathol., 57, 864–873.

Jíménez-Arias, D., Morales-Sierra, S., Silva, P. et al. (2023). Encapsulation with natural polymers to improve the properties of biostimulants in agriculture. Plants, 12, 55. https://doi.org/10.3390/plants12010055

Krieken, W.M.V. der, Rutten, W.B.A.H., Jans, C.G.J.M. (2019). Polyelectrolyte complexes for biocide enhancement. United States Patent, US10342228B2.

Kudasova, D., Mutaliyeva, B., Vlahoviček-Kahlina, K. (2021). Encapsulation of synthesized plant growth regulator based on copper (II) complex in chitosan/alginate microcapsules. Int. J. Mol. Sci., 22, 2663. https://doi.org/10.3390/ijms22052663

Lambrese, Y.S., Illanes, C.O., Ochoa, N.A. (2024). Advancing bacterial endophyte encapsulation in alginate for sustainable agriculture: method comparisons, morphology and viability assessment. J. Clean. Prod., 457, 142473. https://doi.org/10.1016/j.jclepro.2024.142473

Li, M., Yang, X.W., Tian, X.H. et al. (2014). Effect of nitrogen fertilizer and foliar zinc application at different growth stages on zinc translocation and utilization efficiency in winter wheat. Cereal Res. Commun., 42(1), 81–90. https://doi.org/10.1556/CRC.2013.0042

Long, X.X., Yng, X.E., Ni, W.Z. et al. (2003). Assessing zinc tresholds for phytotoxicity and potencial diatery toxicity in selected vegetable crops. Commun. Soil Sci. Plant Anal., 35(9–10), 1421–1434. https://doi.org.sabidi.urv.cat/10.1081/CSS-120020454

Mariano-da-Silva, S., Mottin, F.M., Ducatti, R.D.B. et al. (2025). Effects of copper toxicity on seed germination and seedling growth of *Brassica napus* L. var. *oleifera*. Rev. Gest. Soc. Ambient. – RGSA, 19(2), e010913. https://doi.org/10.24857/rgsa.v19n2-009

Marturano, V., Bizzarro, V., De Luise, A. et al. (2018). Essential oils as solvents and core materials for the preparation of photo-responsive polymer nanocapsules. Nano Res., 11, 2783–2795. https://doi.org/10.1007/s12274-017-1908-5

Marturano, V., Kozlowska, J., Bajek, A. et al. (2019). Photo-triggered capsules based on lanthanide-doped upconverting nanoparticles for medical applications. Coord. Chem. Rev., 398, 213013. https://doi.org/10.1016/j.ccr.2019.213013

McBride, M.B., Bouldin, D.R. (1984). Long-term reactions of copper(II) in a contaminated calcareous soil. Soil Sci. Soc. Am. J., 48, 56–59. https://doi.org/10.2136/sssaj1984.03615995004800010010x

Nepal, A., Antonious, G.F., Bebe, F.N. et al. (2024). Heavy metal accumulation in three varieties of mustard grown under five soil management practices. Environments, 11, 77. https://doi.org/10.3390/environments11040077

Ortega, P., Sánchez, E., Montornes, J.M. et al. (2024). Design and evaluation of microencapsulation technology to reduce the environmental impact of copper fungicides in vineyards. Crop Prot., 176, 106502. https://doi.org/10.1016/j.cropro.2023.106502

Qi, Q., Fan, C., Wu, H. et al. (2023) Preparation of *Trichoderma asperellum* microcapsules and biocontrol of cucumber powdery mildew. Microbiol. Spectr., 11, e05084-22. https://doi.org/10.1128/spectrum.05084-22

Rahman, M., Khatun, A., Liu, L. et al. (2024). *Brassicaceae* mustards: phytochemical constituents, pharmacological effects, and mechanisms of action against human disease. Int. J. Mol. Sci., 25, 9039. https://doi.org/10.3390/ijms25169039

Ramzan, T., Shahbaz, M., Maqsood, M.F. et al. (2023). Phenylalanine supply alleviates the drought stress in mustard (*Brassica campestris*) by modulating plant growth, photosynthesis, and antioxidant defense system. Plant Physiol. Biochem., 201, 107828. https://doi.org/10.1016/j.plaphy.2023.107828

Rather, B.A., Mir, I.R., Masood, A. et al. (2020). Nitric oxide pre-treatment advances seed germination and alleviates copper-induced photosynthetic inhibition in Indian mustard. Plants, 9, 776. https://doi.org/10.3390/plants9060776

Reig-Vano, B., Tylkowski, B., Montané, X. et al. (2021). Alginate-based hydrogels for cancer therapy and research. Int. J. Biol. Macromol., 170, 424–436. https://doi.org/10.1016/j.ijbiomac.2020.12.161

Scher H.B., Shirley I.M., Chen J. et al. 2003. Novel microcapsules. European Patent Office, EP1292386A2.

Shaban, G.M., El-Komy, H.M.A. (2001). Survival and proliferation of alginate encapsulated *Trichoderma* spp. in Egyptian soil in comparison with allyl alcohol soil fumigation. Mycopathologia, 151, 139–146. https://doi.org/10.1023/A:1017968429632

Shaheen, S.M., Tsadilas, C.D., Rinklebe, J. (2013). A review of the distribution coefficients of trace elements in soils: influence of sorption system, element characteristics, and soil colloidal properties. Adv. Colloid Interface Sci., 201–202, 43–56. https://doi.org/10.1016/j.cis.2013.10.005

Staff, R.H., Mecfel-Marczewski, J., Semar et al. (2022). New microcapsules for agricultural applications. United States Patent Publication Aplication, US20220217976A1.

Takahashi, H., Scott, T.K., Suge, H. (1992). Stimulation of root elongation and curvature by calcium 1. Plant Physiol., 98, 246–252. https://doi.org/10.1104/pp.98.1.246

Tylkowski, B., Olkiewicz, M., Montane, X. et al. (2020). 11. Encapsulation technologies in agriculture. In: B., Tylkowski, M., Giamberini, S., Fernandez Prieto (eds.), Microencapsulation. De Gruyter, 287–302. https://doi.org/10.1515/9783110642070-011

Vinceković, M., Jurić, S., Đermić, E. et al. (2017). Kinetics and mechanisms of chemical and biological agents release from biopolymeric microcapsules. J. Agric. Food Chem., 65, 9608–9617. https://doi.org/10.1021/acs.jafc.7b04075

Wen X., Xu P., Tang Y. et al. (2024). Effect of copper on nitrogen uptake, transportation, assimilation processes, and related gene expression in Chinese cabbage [*Brassica campestris* L. ssp. *Chinensis* (L.)] under various nitrate-to-ammonium ratios. Fron. Plant Sci., 15, 1427720. https//doi.org/10.3389/fpls.2024.1427720

Wong, L.W., Fahimizadeh, M., Lee Tan, J.B. et al. (2023). Chapter 11. Polyurea microcapsules as effective carriers for biomedical and agricultural applications. In: P., Pasbakhsh, D., Mohotti, K., Palaniandy et al. (eds.), Polyurea. Elsevier, pp. 191–202. https://doi.org/10.1016/B978-0-323-99450-7.00019-8

Woźniak-Budych, M., Staszak, K., Wieszczycka, K. et al. (2024). Microplastic label in microencapsulation field – consequence of shell material selection. J. Hazar. Mater., 465, 133000. https//doi.org/10.1016/j.hazmat.2023.133000

Xin, X., Zhao, F., Rho, J.Y. et al. (2020). Use of polymeric nanoparticles to improve seed germination and plant growth under copper stress. Sci. Total Environ., 745, 141055. https://doi.org/10.1016/j.scitotenv.2020.141055

Yu, H., Li, C., Yan, J. et al. (2023). A review on adsorption characteristics and influencing mechanism of heavy metals in farmland soil. RSC Advances, 13, 3505. https://doi.org/10.1039/d2ra07095b

Zhao, X., Guo, Y., Papazoglou, E.G. (2022). Screening flax, kenaf and hemp varieties for phytoremediation of trace element-contaminated soils. Ind. Crops Prod., 185, 115121. https://doi.org/10.1016/j.indcrop.2022.115121

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REVIEW PAPER

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DYNAMICS OF *Epichloë*-GRASS RELATIONSHIPS: AN ANALYSIS OF THE DUALISTIC NATURE

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ABSTRACT

This review provides a comprehensive discussion of *Epichloë* fungi, grass endophytes that form deeply integrated symbioses, including the taxonomy and morphology of these organisms, along with the evolution of their classification based on molecular data. The complex nature of their symbiotic interactions is detailed, ranging from mutualistic protection against biotic and abiotic stresses (e.g., drought, silicon accumulation, enhanced photosynthesis) to potential livestock toxicity resulting from alkaloid production. We present analyses of the fung's life cycles, including vertical (mutualistic) and horizontal (pathogenic) transmission, and the role of hybridization in shaping alkaloid profiles. The production of alkaloids (lolines, indole-diterpenes, ergot alkaloids, peramine) and their biological effects are discussed. We also highlight the dynamics of host specificity and coevolution. Furthermore, detection and characterization methods are presented, emphasizing molecular techniques like PCR and microsatellite analysis for rapid and precise strain identification. Finally, the significant agronomic and ecological implications of *Epichloë* endophytes are addressed, underscoring their potential in sustainable agriculture through the development of "safe" and effective strains. New topics included in the review include information on the use of modern molecular markers for rapid and precise strain identification. Furthermore, the review highlights the extended benefits of endophyte presence for the host through silicon accumulation and manipulation of photosynthesis, as well as a new approach to alkaloid biosynthesis through domain shuffling, which allows for genetically based prediction of alkaloid profiles.

Key words: alkaloids, biotic and abiotic stresses, *Epichloë*, grass endophyte

INTRODUCTION

Epichloë fungi are a group of endophytes that form complex and long-lasting symbiotic relationships with various grass species [Schardl et al. 2004, Christensen 2008]. These microscopic organisms colonize the intercellular spaces of the host plant, spending most of their life cycle within it, typically without causing visible external symptoms [Siegel et al. 1989, Clay 1990]. This hidden presence, coupled with their systemic growth throughout the plant, from leaves to seeds, indicates a highly evolved and deeply integrated symbiotic relationship. The fungus must possess sophisticated mechanisms to avoid or suppress host defense responses, enabling prolonged coexistence [Mathew et al. 2022, Zhao et al. 2025]. This deep integration is a prerequisite for the observed systemic benefits, as the fungus must be widely distributed within the plant to exert its protective effects in its tissues [Schardl 1996, Siegel and Bush 1996, Grabka et al. 2022].

The dualistic nature of *Epichloë* species is one example of biological complexity in nature, which can be considered on two main levels: ecological (their relationship with the plant) and agricultural (their impact on animals). This stems from the diversity of alkaloids they produce, which, on the one hand, have a beneficial effect on the plant by increasing its resistance to biotic and abiotic stresses (drought, salinity, heavy metals, fungal diseases,

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and pests). At the same time, others, such as ergovaline and lolitrem, are highly harmful to livestock, negatively impacting their health, productivity, and fertility.

The role of *Epichloë* fungi extends far beyond mere coexistence, making them key factors in the biological protection of grasses. They provide resistance to a range of biotic factors, such as nematodes, insects, wild animals, and grazing livestock [Bastias et al. 2017, Karpyn Esqueda et al. 2017, Caradus 2023], as well as to abiotic stresses, including drought [Schardl 1996, Rodriguez and Redman 2008, Nagabhyru et al. 2022]. Their influence extends to modulating grassland ecosystems, impacting both aboveground and belowground processes [Vikuk et al. 2019, Wang et al. 2020, Wang et al. 2024]. However, despite these significant benefits, the interactions of *Epichloë* with grasses present a complex challenge. Some fungal strains produce compounds that are toxic to livestock, creating a paradox where the same symbiont can be both immensely beneficial and potentially harmful [Caradus and Johnson 2020, Fredell et al. 2025]. This duality is a primary driver of research and commercial applications, aiming to harness the benefits while minimizing the risks. If a biological agent has both highly beneficial and highly detrimental effects, it poses a complex challenge for agricultural management. This inherent tension demands a scientific approach focused on understanding the mechanisms of both benefits and harms, leading to strategies for selective breeding or engineering of "safe" and effective strains [Schardl and Leuchtmann 2005, Saikkonen et al. 2016, Pozo et al. 2021].

The aim of this review is to comprehensively discuss *Epichloë* fungi, grass endophytes that form deeply integrated symbioses, taking into account their taxonomy, evolution, morphology, and the complex nature of their symbiotic interactions. Furthermore, it explores additional host benefits beyond alkaloid production, the dynamics of host specificity and coevolution, and methods for detecting and characterizing these fungi. The paper also delves into the significant agronomic and ecological implications of *Epichloë* endophytes, including their potential applications in sustainable agriculture.

TAXONOMY, MORPHOLOGY AND CHARACTERIZATION OF *Epichloë* FUNGI

Epichloë fungi belong to the phylum *Ascomycota*, order *Hypocreales,* and family *Clavicipitaceae* [White 1994, 1997, White and Reddy 1998, Du et al. 2024]. These are grass endophytes that spend most of their life cycle within the intercellular spaces of their host plant, typically without causing visible external symptoms [Siegel et al. 1985, White and Cole 1985, Gams et al. 1990, Wilson 1995]. In laboratory conditions, on solid media, their morphological characteristics such as colony color, diameter, and growth rate are thoroughly examined, often by measuring colony diameter using the "cross" method [Wang et al. 2025].

The endophytes are relatively slow-growing fungi and require specific nutrient-rich media for successful isolation and characterization. The most common universal medium recommended for initial isolation and observing colony morphology is potato dextrose agar (PDA). However often to stimulate sporulation in certain strains is using cornmeal agar (CMA). Additionally is using also malt extract agar (MEA), which provides a rich source of nitrogen and carbon, and is suitable for long-term maintenance of cultures [Stone et al. 2000].

The history of *Epichloë* taxonomy exemplifies the evolution of fungal classification, driven by advacements in molecular methods [Schardl et al. 2004]. Initially, anamorphic (asexual) grass endophytes were classified under the genus *Acremonium*, then reclassified to *Neotyphodium* [Glenn et al. 1996]. However, based on comprehensive phylogenetic reviews and DNA sequence data, such as 18S ribosomal DNA, beta-tubulin gene (*tubB*), and translation elongation factor 1-alpha gene (*tefA*), *Neotyphodium* species were re-incorporated into the genus *Epichloë* [Schardl et al. 2004, Leuchtmann et al. 2014]. This reclassification reflects a shift from classification based solely on phenotypic (morphological) characteristics to a system based on genetic and evolutionary relatedness. Traditional mycological classification often relied on observable reproductive structures. Asexual fungi (anamorphs) were grouped separately from their sexual counterparts (teleomorphs) if the sexual stage was unknown or absent. With the advent of DNA sequencing, it became possible to trace evolutionary lineages more accurately. This revealed that many *Neotyphodium* species were, in fact, asexual forms or hybrids of known *Epichloë* species. This reclassification is not merely a name change but reflects a fundamental shift in our understanding of their biological identity and evolutionary relationships, emphasizing genetic continuity over reproductive strategy. An example is the endophyte from *Ammophila breviligulata* Fern. initially identified as *Acremonium typhinum* var. *ammophilae* White and Morgan-Jones. and subsequently *Epichloë typhina* var. *ammophilae* (White and Morgan-Jones) White, which was phylogenetically identified as a member of *E. amarillans* White [Drake et al. 2018].

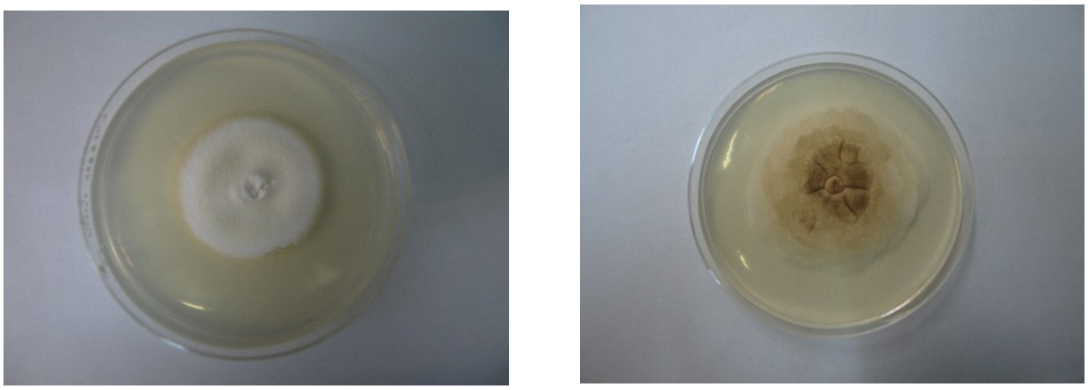
Epichloë fungi colonize a wide range of grasses, predominantly from temperate zones, encompassing numerous species. These include fescues such as meadow fescue (*Festuca pratensis* Huds.), tall fescue (*Festuca arundi-*

nacea Schreb.), red fescue (*Festuca rubra* L.), sheep fescue (*Festuca ovina* L.), hair fescue (*Festuca capillata* Lam.), and giant fescue (*Festuca gigantea* L.). These endophytes also occur in ryegrasses: perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) [Christensen et al. 1997, Schardl et al. 2004, Scott et al. 2012, Leuchtman et al. 2014, Becker et al. 2016]. The host list further includes bluegrasses like Kentucky bluegrass (*Poa pratensis* L.) and wood bluegrass (*Poa nemoralis* L.), timothy (*Phleum pratense* L.), tufted hairgrass (*Deschampsia caespitosa* (L.) P.B.), various *Agrostis* species (e.g., redtop – *Agrostis gigantea* Roth), orchardgrass (*Dactylis glomerata* L.), and alkali grass (*Puccinellia distans* (Jacq.) Parl.). Additionally, *Epichloë* has been found in grasses such as reed grasses (*Calamagrostis arundinacea* L. (Roth.), *C. villosa* (Chaix) J.F.Gmel., *C. varia* (Schrad.) Host, *C. purpurea* (Trin.) Trin.), as well as *Bromus erectus* Huds., *B. benekenii* (Lange) Trimen, *B. ramosus* Huds., *Elymus repens* (L.), *E. tsukushiensis* Honda, *Hordelymus europaeus* (L.) Jess. ex Harz, *H. brevisubulatum* (Trin.) Link, *Leymus chinensis* (Trin.), soft brome (*Holcus mollis* L.), and velvet grass (*H. lanatus* L.) [Clay and Schardl 2002, Schardl et al. 2004, Schardl 2010, Luna-Fontalvo et al. 2025].

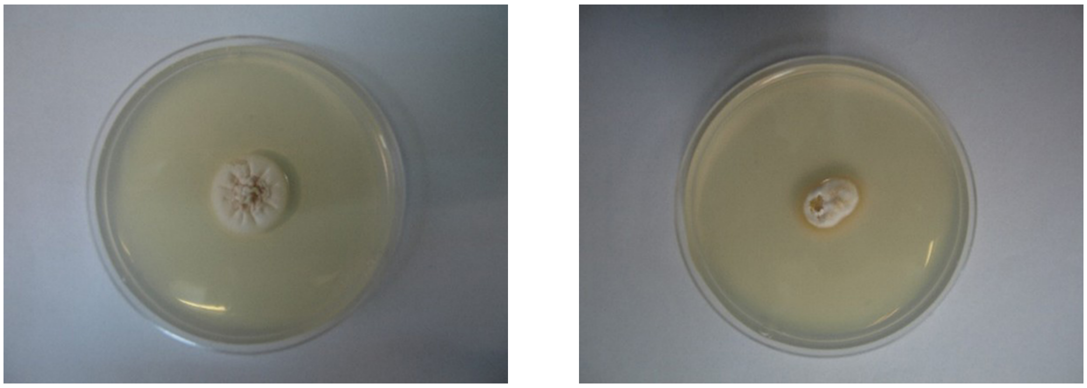
The morphological characteristics of *Epichloë* fungi in culture, such as colony color, diameter, growth rate (Figure 1), conidia size, and conidiogenous cell length, exhibit significant interspecific variability [Schardl et al. 2004, Leuchtman et al. 2014].

Although these properties are not sufficient for definitive taxonomic grouping, they are crucial for initial isolation, identification, and understanding of the physiological diversity within the genus [Schardl et al. 2004]. Wang et al. [2025] observed clear differences in growth rate and conidia sizes among various *Epichloë* species, which is utilized in laboratory research for strain characterization. This means that morphological analysis, combined with molecular data, provides a more complete picture for strain identification and characterization, aiding in the selection of specific strains for agricultural applications or in understanding their ecological niches [Leuchtman et al. 2014, Du et al. 2024]. This also highlights the need for a multi-faceted approach to identification.

Figure 1. Appearance of pure *Epichloë* cultures isolated from individual grass species after 3 weeks of incubation (photo B. Wiewióra)



Festuca rubra *Festuca pratensis*



Lolium perenne *Poa pratensis*

METHODS OF DETECTION AND CHARACTERIZATION

Detecting and characterizing *Epichloë* fungi in their host plants has evolved from traditional techniques to advanced molecular methods, allowing for precise and rapid identification. Early in-plant detection methods included histological staining, which allowed visualization of fungal hyphae [Latch and Christensen 1985]. Immunological assays, such as ELISA and tissue-print immunoblot, were also employed [Gwinn et al. 1991, Hill et al. 2002]. More recently, PCR-based methods have become dominant for endophyte detection and quantification, both in culture and in plants, due to their speed, sensitivity, and specificity [Doss and Welty 1995, Dombrowski et al. 2006, Sharma et al. 2020]. The evolution from histological staining and immunological assays to sophisticated PCR-based methods, especially microsatellite analysis, marks a critical shift toward precise diagnostics in *Epichloë* research. This allows for rapid, sensitive, and highly specific strain identification, which is paramount for risk management in agriculture (e.g., identifying toxic strains) and for targeted breeding programs. Older methods were often slower, less specific, or required culturing the fungus first, which could take weeks. In contrast, molecular methods can directly detect the fungus in plant tissue, providing quick results. This speed and precision are essential in agricultural settings where rapid identification of endophyte strains (and their associated alkaloid profiles) is necessary for decisions regarding livestock grazing or seed certification [Takach and Young 2014].

- Randomly Amplified Polymorphic DNA (RAPD): this is a PCR-based method used for detection and quantification [Moon et al. 2000].
- Microsatellite Loci Analysis (Microsatellite-based PCR Fingerprinting): this method leverages the polymorphic properties of microsatellite loci (short tandem repeats) with the speed and sensitivity of PCR. It enables precise determination of amplified product size through automated analysis using fluorochrome-labeled primers and laser scanners. A reference database of allele sizes can be created, allowing for distinguishing endophyte groups at the level of known isozyme groups. The assay is specific for *Epichloë* DNA in the plant, even in complex mixtures of plant DNA, and can be expedited with specific DNA extraction methods [Takach et al. 2012]. Certain loci (e.g., B11, B9, B10) are particularly informative, allowing for differentiation of multiple patterns and identification of hybrid endophytes due to the presence of multiple alleles. Multiplex PCR can be used to amplify multiple loci simultaneously, saving reagents and time. Automated analysis provides high detection sensitivity, safer handling, faster data collection, simplified analysis of hybrid endophytes, and data generation as DNA fragment size for interlaboratory comparisons. The ability to combine genetic characterization (e.g., presence/absence of alkaloid biosynthesis genes via PCR) with precise strain identification (via microsatellite analysis) allows for a predictive approach to toxidrome risk assessment [Schardl et al. 2013b]. This means that farmers and breeders can know the potential toxicity of a grass-endophyte combination without having to wait for symptoms in livestock, facilitating proactive management. If a specific *Epichloë* strain can be identified and its genetic potential for alkaloid production is known, it's possible to predict whether it will cause "fescue toxicosis" or "ryegrass staggers" before any animals are affected, which has enormous economic and animal welfare benefits.
- Phylogenetic Analysis: the phylogenetic position of *Epichloë* isolates can be investigated using specific genes such as *tefA* and *actG* or *tubB* [Craven et al. 2001].

While Microsatellite Loci Analysis enables the identification of hybrid endophytes through the presence of multiple alleles, the rapid evolution of genomics has introduced tools that provide unparalleled resolution. Especially crucial for analyzing the complex, heteroploid genomes often found in *Epichloë* species Long-Read Sequencing (LRS), Pangenome Frameworks or SNP Assays and Digital PCR (dPCR) [Schreiber et al. 2024, Huggett and Whale 2013]. Equally important is comprehensive alkaloid profiling based on multiplex LC-MS/MS (liquid chromatography with tandem mass spectrometry) or LC-HRMS (liquid chromatography with high-resolution mass spectrometry) [Rudolph et al. 2018, Berry et al. 2019, Vassiliadis et al. 2019]. These modern genomic and chemical tools deliver the speed, precision, and comprehensive resolution required to manage the economic and animal welfare aspects of the grass-endophyte symbiosis effectively today.

Epichloë-GRASS SYMBIOSIS: A CONTINUUM OF INTERACTIONS

The nature of the symbiosis between *Epichloë* and grasses is variable, ranging from antagonism to mutualism, depending on prevailing selective pressures, environmental conditions, and the specific genotypes of the fungus and host. Their mode of interaction is highly dependent on the surrounding situation, environment, or specific conditions [Saikkonen et al. 1998, Faeth and Sullivan 2003, Saikkonen et al. 2016]. Antagonistic aspects often arise from the fungus's sexual cycle, during which it produces stromata that can cause abortion of host inflorescences, leading to "choke disease," which is considered the pathogenic end of the continuum [Saikkonen et al. 1998, von Cräutlein et al. 2021]. Conversely, mutualistic aspects include protective fungal alkaloids and other benefits for systematically infected plants. Host benefits are largely associated with defensive mutualism, resulting from bioactive fungal-derived alkaloids [Bush et al. 1997, Schardl et al. 2004, Cadus and Johnson 2020].

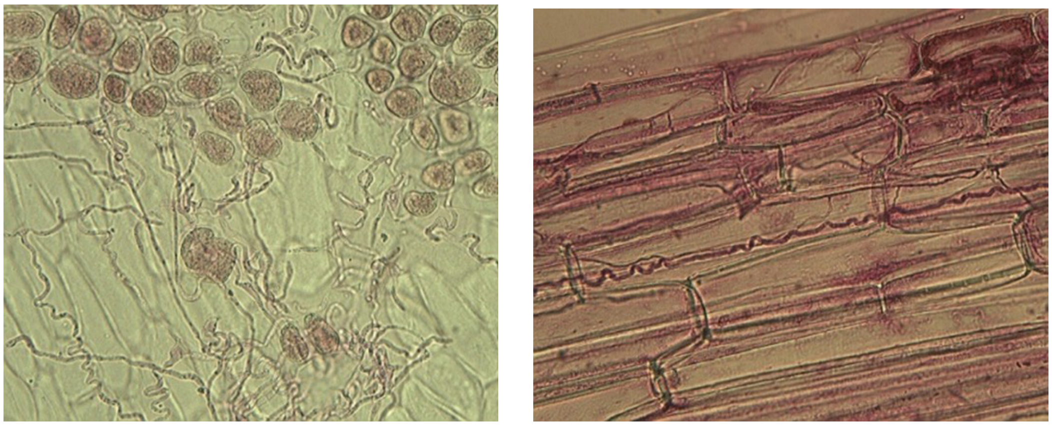
The symbiosis is characterized by a high degree of integration, involving the systemic growth of the fungus in the host's aerial parts, including leaf sheaths, inflorescences, ovules, and seed embryos [Clay 1990]. The fungus grows intercellularly within the host tissues. This intimate relationship requires the fungus to adapt to gain access to the host plant's interior, likely by suppressing the plant's recognition and defense responses that would normally halt harmful fungal infections [Saikkonen et al. 1998]. The necessity for the fungus to suppress host recognition and defense responses indicates a continuous process of reciprocal evolutionary adaptation [Schardl et al. 2012]. The host attempts to detect and defend itself against fungal invaders, while the endophyte evolves mechanisms to evade or modulate these defenses to establish a stable, asymptomatic infection. This suggests a complex molecular dialogue between host and symbiont [Lee et al. 2021]. Plants have developed sophisticated immune systems to detect pathogens [Jones and Dangl 2006]. For an endophyte to live asymptomatically within a plant, it must either be "invisible" to the plant's defense mechanisms or actively suppress them [Saikkonen et al. 1998, Redman et al. 2002, Rodriguez et al. 2009]. This is not a passive process; it represents a finely tuned co-adaptation where the fungus has evolved specific strategies to bypass or disarm host immunity, enabling the establishment of a symbiotic rather than a pathogenic state, which underscores the molecular sophistication of this interaction [Redman et al. 2002, Jones and Dangl 2006, Dodds and Rathjen 2010, Schardl et al. 2012].

Epichloë fungi exhibit various life cycles, categorized primarily by transmission methods, reflecting a fundamental evolutionary trade-off.

Vertical transmission (asexual forms)

These forms, formerly known as *Neotyphodium*, are obligate endophytes [Schardl et al. 2004, Leuchtman et al. 2014]. They are clonally and vertically transmitted from the mother plant to its progeny via host seeds [Caradus and Johnson 2020]. The fungus penetrates the developing ovule and eventually the embryo and endosperm of mature seeds [Philipson and Christey 1986, Rodriguez et al. 2009] (Figure 2). Asexual *Epichloë* species typically do not produce sexual spores (hymenium) and rarely produce asexual spores (conidia), except under axenic culture conditions in the laboratory [Clay 1990, Schardl et al. 2004]. Many asexual strains are interspecific hybrids, often resulting from hybridization events between two different *Epichloë* species. They usually possess heteroploid genomes [Charlton et al. 2014]. This mode of transmission is associated with high host specificity and often favors the formation of beneficial (mutualistic) associations [Zabalgogeazcoa 2008]. If a microorganism survival relies solely on the host's ability to reproduce and transmit it through seeds, then any trait that harms host reproduction (such as inflorescence strangulation) would be strongly eliminated. This creates strong evolutionary pressure for mutualism [Saikkonen et al. 1998].

Figure 2. Characteristic hyphae in the seed (left) and in the plant (right) (photo B. Wiewióra)

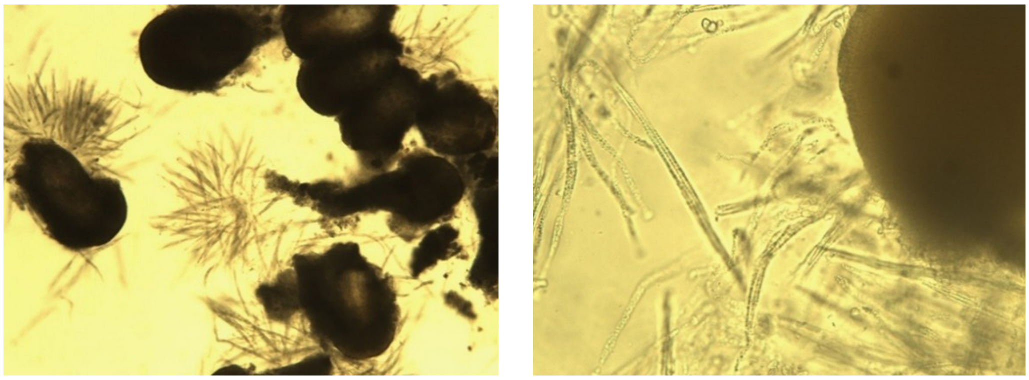


Horizontal transmission (sexual forms)

Sexual *Epichloë* species produce fruiting bodies called stromata, which encircle the immature host inflorescence and halt its development, causing "choke disease" [White 1997] (Figure 3). These are transmitted horizontally via sexual spores (ascospores) [Schardl et al. 2004] (Figure 4). However, this is not the only possibility of horizontal transmission, because Wiewióra et al. [2015] found in their work that horizontal transmission of mycelia is possible also through the transfer of hyphae during mowing and trampling of grass.

Figure 3. Stromata on meadow timothy (left) and sheep's fescue (right) (photo: B. Wiewióra)

Figure 4. Asci and ascospores of *Epichloë* sp. ×100 (left) and ×400 (right) (photo: B. Wiewióra)



Fertilization requires the dissemination of spermatia (male gametes) onto unfertilized stromata of the opposite mating type [Clay and Schardl 2002]. This process is vectored by phylogenetically distinct flies from the family *Anthomyiidae* (e.g., *Botanophila* spp., formerly *Phorbia* spp.), which are attracted by fungal volatile compounds, feed on the perithecial tissue containing spermatial spores, and lay their eggs on the stromata [Bultman et al. 1998]. Horizontally transmitted *Epichloë* fungi typically possess haploid genomes [Schardl and Craven 2003]. This strategy can be considered pathogenic due to host castration [Chung et al. 1997]. Conversely, if a microorganism can spread independently via spores, it has less "stake" in the reproductive success of a single host, allowing for more exploitative or pathogenic strategies. This distinction is crucial for understanding the ecological roles and agricultural applications of different *Epichloë* strains.

Pleiotropic species

Some *Epichloë* species e.g. *E. typhina* exhibit a mixed strategy, undergoing both sexual (horizontal) and asexual (vertical) cycles [Schardl et al. 2004]. The frequent occurrence of asexual *Epichloë* strains as interspecific hybrids is a significant evolutionary mechanism [Tsai et al. 1994]. This hybridization allows for the combination of genetic material from different sexual species, potentially leading to new alkaloid profiles and host associations that are highly beneficial and vertically transmissible, without the "cost" of the sexual cycle (choke disease). Sexual reproduction allows for genetic recombination and diversity. If asexual strains are hybrids, they gain genetic diversity from their sexual ancestors without needing to undergo their own sexual cycle. This can lead to new gene combinations, including those responsible for alkaloid biosynthesis, potentially creating strains with unique defensive capabilities that are highly adapted for mutualistic, vertical transmission. This explains how "novel endophytes" with desired traits can arise and be selected for agricultural applications.

ALKALOID PRODUCTION AND BIOLOGICAL EFFECTS

Epichloë species are known for producing a range of secondary metabolites, primarily alkaloids, which are crucial for defensive mutualism with their grass hosts. The production of these alkaloids depends on the specific *Epichloë* species and can be modified by environmental conditions and host genotype. Alkaloid diversity is largely determined by the presence or absence of specific alkaloid biosynthesis genes [Moore et al. 2015]. However, research has also shown that structural diversity in secondary metabolites, such as pyrrolopyrazines, can arise through mechanisms like nonenzymatic cyclization and domain shuffling in nonribosomal peptide synthetetases [Berry et al. 2019]. This allows for predicting synthesized alkaloids based on genetic studies, which is much more efficient than chemical analysis alone. This genetic understanding is fundamental for developing "novel endophytes" that produce beneficial, insect-deterring compounds without vertebrate toxins, and serves as a powerful selection tool.

There are four main classes of bioactive alkaloids (Table 1):

– lolines (saturated 1-aminopyrrolizidines)
– indole-diterpenes
– ergot alkaloids
– peramine (pyrrolopyrazine alkaloid).

The differentiation of alkaloid classes into those primarily toxic to insects (lolines, peramine) and those also toxic to vertebrates (ergot alkaloids, indole-diterpenes) suggests an evolutionary specialization of defense mechanisms (Table 2). This means that *Epichloë* has evolved to counteract a wide range of herbivores, but with varying degrees of specificity and collateral impact. If a single defense mechanism worked against all herbivores, there would be no need for multiple alkaloid classes. The existence of distinct classes with different target specificities (insects vs. vertebrates) also presents a challenge for agriculture: selecting strains that maintain a broad spectrum of insect defense while eliminating or minimizing "collateral damage" to livestock, which are unintended targets of some of these defense mechanisms.

Table 1. Classes of alkaloids produced by *Epichloë* endophytes and their biological effects on herbivores and livestock

Alkaloid class	Examples	Effects on livestock	Insecticidal properties
Lolines	N-formylloline (NFL), N-acetylloline (NAL), N-acetylnorloline (NANL)	no known mammalian toxicity	repellent and toxic to insects (e.g., Argentine stem weevil, black cutworm, aphids, scarab larvae)
Indole-diterpenes	lolitrem B, epoxyjanthitrems, paxilline, terpendoles	"ryegrass staggers" – neurotoxins, tremors, affect muscle coordination, respiration, heart rate	anti-insect properties (e.g., Argentine stem weevil, root aphids)
Ergot alkaloids	ergovaline, ergotamine, ergonovine, agroclavine, lysergic acid	"fescue toxicosis" – inability to regulate temperature, vasoconstriction, heat stress, reduced prolactin	anti-insect properties (e.g., black cutworm, Argentine stem weevil)
Peramine	peramine, pyrrolopyrazine	no known mammalian toxicity	insect repellent (e.g., Argentine stem weevil, aphids)

Alkaloid profiles can differ even within the same *Epichloë* species, which is due to the genetic diversity of the alkaloid gene clusters, and the host plant can moderately influence the level of alkaloid production by the endophyte, although to a lesser extent than the genetics of the fungus [Talamantes et al. 2025]. Furthermore, the distribution of alkaloids within the plant can vary and doesn't always correlate with the fungal hyphae's distribution [Tian et al. 2020]. Alkaloid concentrations are usually highest in the seeds. In the shoots/roots, the distribution can be different, e.g. peramine and lolitrem B concentrations are often higher in the shoots, while ergovaline may be more evenly distributed [Vassiliadis et al. 2023, Realini et al. 2024].

Table 2. *Epichloë* species in grasses and the alkaloids they produce

Species of *Epichloë*	Host plant	Alkaloids produced	Reference
E. amarillans	*Agrostis hyemalis*	NANL (N-acetylnorloline), PER (peramine)	Schardl et al. 2013a
E. amarillans	*Agrostis hyemalis*	ERV (ergovaline), PER (peramine)	Schardl et al. 2013a
E. baconii	*Calamagrostis villosa*	PER (peramine)	Schardl et al. 2013a
E. brachyelytri	*Brachyelytrum erectum*	CC (chanoclavine), AcAP (1-acetamidopyrrolizidine), PER (peramine)	Schardl et al. 2013a
E. bromicola	*Bromus erectus*	no alkaloids detected	Schardl et al. 2013a
E. bromicola	*Bromus tomentellus*, *Melica persica*, *Thinopyrum intermedium*	LOL (loline alkaloids)	Schardl et al. 2025
E. coenophiala	*Festuca arundinaceum*	Ergovaline alkaloids (e.g. ergovaline), peramine, lolines	Emile et al. 2000 Vazquez de Aldana et al. 2001 Żurek et al. 2010 Wiewióra 2011 Leuchtmann et al. 2014
E. elymi	*Elymus virginicus*	CC (chanoclavine), PER (peramine)	Schardl et al. 2013a
E. festucae	*Festuca rubra*, *Lolium giganteum*	ERV (ergovaline), NFL (N-formylloline)	Leuchtmannn et al. 2000 Żurek et al. 2010 Wiewióra 2011 Schardl et al. 2013a
E. festucae	*Festuca trachyphylla*	ERV (ergovaline), LTB (lolitrem B), PER (peramine)	Schardl et al. 2013a
E. festucae var. lolii	*Lolium perenne*	LTB (lolitrem B), PER (peramine), ergovaline alkaloids	Oliveira et al. 1997 Cagaš et al. 1999 Żurek et al. 2010 Wiewióra 2011 Schardl et al. 2013a
E. funkii	*Achnatherum robustum*	CC (chanoclavine), TD (tremorgenic indole-diterpenes), PER (peramine)	Schardl et al. 2013a
E. gansuensis	*Achnatherum inebrians*	PAX (paxilin)	Schardl et al. 2013a
E. gansuensis var. *inebrians*	*Achnatherum inebrians*	LAH (lysergic acid α-hydroxyethylamide), ergonovine, ergine	Schardl et al. 2013a
E. glyceriae	*Glyceria striata*	ERV (ergovaline), AcAP (1-acetamidopirolizidine)	Schardl et al. 2013a
E. mollis	*Holcus mollis*	ERV (ergovaline), PER (peramine)	Schardl et al. 2013a
E. poae	*Poa nemoralis*	no alkaloids detected	Schardl et al. 2013a
E. poae	*Poa nemoralis*	ERV (ergovaline)	Schardl et al. 2013a

Species of *Epichloë*	Host plant	Alkaloids produced	Reference
E. typhina	*Lolium perenne*	PER (peramine)	Schardl et al. 2013a
E. uncinata	*Festuca pratensis*	loline compounds	Cagaš et al. 1999 Żurek et al. 2010 Wiewióra 2011 Vikuk et al. 2019
Epichloë sp.	*Festuca ovina* agg.	indole-diterpene intermediates	Vikuk et al. 2019
Epichloë sp.	*Achnatherum robustum*	ergonovine, ergine	Guerre 2015
Epichloë clarkii	*Holcus lanatus*	No data	Leuchtmann et al. 2014

It's worth noting that alkaloid expression levels can be quantitatively modified by environmental factors. They are strongly expressed in the plant, but at very low levels or not at all in axenic cultures (without the presence of other organisms) [Aniszewski 2007].

HOST BENEFITS BEYOND ALKALOID PRODUCTION

Beyond the well-documented chemical defense mechanisms, the symbiosis with *Epichloë* provides grass hosts with a range of other physiological and developmental benefits, increasing their overall fitness and resilience.

Epichloë endophytes can provide host plants with resistance to various abiotic stresses, including drought tolerance, salt tolerance, and waterlogging [Rodriguez and Redman 2008, Decunta et al. 2021]. A particularly interesting mechanism is silicon (Si) accumulation. The presence of *Epichloë* significantly increases silicon concentration in the leaves of host grasses; for example, in tall fescue this increase is at least 31%, and in perennial ryegrass it is 47% for specific strains [Cibils-Stewart et al. 2020]. This increase is independent of plant growth and positively correlates with endophyte colonization. Potential mechanisms for increased Si uptake may include increased transpiration rates by symbionts, a direct impact on plant aquaporins, or alterations to endogenous host defense pathways (e.g., the jasmonic acid pathway) that promote Si uptake. Morphological changes, such as an increased number of vascular bundles, may also contribute to this phenomenon. Interestingly, silicon supply can, in turn, increase *Epichloë* colonization (e.g., by over 60% in tall fescue), likely by creating additional niches or increasing carbon availability for the fungus. The discovery that *Epichloë* increases host silicon accumulation, and silicon in turn enhances *Epichloë* colonization, reveals a sophisticated positive feedback loop. This suggests a deeper level of mutualism where the fungus not only provides direct benefits but also actively modifies the host physiological environment in a way that optimizes its own growth and survival within the plant [Ma and Yamaji 2006]. This goes beyond a simple "fungus gives, plant receives" model. If the fungus helps the plant acquire a resource (Si) beneficial for the plant structural integrity and defense, and this resource also benefits fungal colonization, it indicates a coevolutionary mechanism that strengthens the symbiosis itself. This is a more complex interaction than just the production of defensive compounds; it is about active shaping the host's internal environment for the mutual benefit of both partners, making the symbiosis more resilient and effective.

The fungal endophyte *Epichloë typhina* significantly improves the growth, PSII photochemistry, and carbon assimilation efficiency of its host, orchardgrass (*Dactylis glomerata*) [Rozpądek et al. 2015]. These mechanisms include increased biomass, increased electron transport rate (ETR), photochemical quenching coefficient (qL), and effective quantum yield of PSII (Y(II)). *E. typhina* also enhances net photosynthesis and carboxylation rates and lowers the CO_2 compensation point. Increased stomatal conductance is also observed. Furthermore, in *Epichloë*-colonized plants, a higher total chlorophyll concentration (approximately 33% increase) and a twofold increase in chlorophyll b concentration were found. The increased abundance of PSII (D1, Lhcb3) and PSI (PsaC, Lhca2) proteins indicates a higher photosynthetic capacity. The elevated activity of NADPH-malate dehydrogenase (NADPH-MDH)—a sixfold increase—helps in thylakoid lumen deacidification and maintaining an appropriate ATP/NADPH ratio, potentially providing additional energy resources for the plant or its fungal partner [Rozpądek et al. 2015]. The detailed mechanisms by which *Epichloë typhina* enhances photosynthesis, including increased chlorophyll b, PSII/PSI proteins, and NADPH-MDH activity, indicate that the fungus is not merely a passive recipient of host photosynthates. Instead, it appears to actively influence host metabolism to increase overall carbon assimilation. This suggests a sophisticated level of metabolic integration and manipulation by the endophyte to ensure a greater supply of resources, not only for itself but also to enhance the host's overall fitness. For an endophyte that relies on host-assimilated carbon, increasing host photosynthetic efficiency is a highly beneficial strategy. The specific increases in light-harvesting complexes and enzymes involved in carbon assimilation and energy distribution suggest a targeted metabolic adaptation of the plant, likely induced by the fungus. This is a powerful example of how a symbiont can directly improve its host's fundamental energy production capacity, supporting the "increased energy demand" of the symbiosis while simultaneously leading to higher host biomass [Rozpądek et al. 2015, Sarkar et al. 2021]. The positive role of endophytes in disease resistance is demonstrated also in studies by Fardella [2024], whose results allowed the identification of a specific antifungal protein called Efe-AfpA. Expression studies confirmed the key role of this protein in the mechanism of endophyte-induced resistance. The characterization of a number of other proteins homologous to Efe-AfpA in various fungal species indicates the existence of enormous, as yet untapped potential in the development of modern methods of plant disease control.

Beyond specific stress tolerances, *Epichloë* symbioses generally enhance the host plant's fitness, leading to increased growth, vigor, and competitive advantage [Clay 1990, Schardl et al. 2004]. Selected asexual *Epichloë* strains can increase plant biomass and seed yield [Malinowski and Belesky 2019, Gundel et al. 2020].

Despite enormous progress in research on the symbiosis between grasses and fungi of the genus *Epichloë,* the influence of these microorganisms on the dynamics of plant communities and soil processes remains one of the least understood areas of endophyte ecology. Most analyses to date have focused on direct host protection from biotic (herbivory) and abiotic (drought) stress, neglecting subtle but crucial indirect mechanisms. However, *Epichloë* has been shown to indirectly affect the rhizosphere. Although this fungus does not physically colonize root tissues, it radically alters its biochemistry by inducing changes in the transport of assimilates to roots, which influences the quantity and chemical composition of root exudates. These changes may selectively promote specific groups of rhizosphere bacteria, altering the structure of the soil microbiome [Zhong et al. 2022]. One of the most controversial aspects is the impact of *Epichloë* on mycorrhizal fungi (AMF). Some studies suggest that aboveground endophytes may compete with AMF for carbon resources, while others indicate synergy in increasing drought resistance [Omacini et al. 2012, Li et al. 2018].

HOST SPECIFICITY AND COEVOLUTIONARY DYNAMICS

The fitness of the *Epichloë* symbiont and its grass host are intimately linked, presumably leading to their coevolution towards specialization and mutually beneficial cooperation [Clay and Schardl 2002, Schardl et al. 2004]. This coevolution involves reciprocal interactions that often lead to specialization. The symbiosis is highly integrated, involving the mutual exploitation and manipulation of morphological, physiological, and life cycle traits to enhance the fitness of the "symbiote" [Clay 1988].

Host specificity is primarily maintained through the vertical transmission of the microbial partner from the host plant to its progeny via seeds [Clay 1990]. This ensures a stable interaction between the fungal genotype and the host lineage. A loss of horizontal spread (horizontal transmission) is often associated with increased genetic host specificity [Moon et al. 2002, Moon et al. 2004, Kuldau and Bacon 2008]. The intimate relationship requires the fungus to adapt to gain access to the host plant interior, likely by suppressing the host recognition and defense responses that would normally halt harmful fungal infections [Schardl et al. 2004]. The clear mention of "genetic incompatibility" limiting endophyte-grass associations is a crucial observation for both ecological understanding and agricultural applications [Christensen 1995]. This means that simply having the right fungal and grass species is insufficient; specific genotypes must be compatible for a stable and beneficial symbiosis to form and persist. This explains the challenges associated with artificially infecting non-hosts and the need for careful strain-to-cultivar matching in commercial products. If every *Epichloë* strain could infect every grass, the system would be much simpler. The concept of genetic incompatibility suggests that there are specific recognition or compatibility genes on both sides that must match for the symbiosis to succeed. This is a significant barrier to horizontal gene transfer or a broad host range, reinforcing the idea of specialization. For agriculture, it means that developing new *Epichloë*-grass associations is not trivial and requires extensive screening and breeding to find compatible partners. Asexual species, often formed through hybridization, have an even more limited host range and have coevolved towards one or a few grass species, forming a mutualistic relationship [Tsai et al. 1994].

The benefits for *Epichloë* species and their grass hosts are rarely symmetrical, and the symbiosis can range from antagonistic to mutualistic [Clay and Schardl 2002]. Conflicting selective pressures likely lead to their destabilization. For example, when pleiotropic and antagonistic *Epichloë* species enter their sexual cycle, they can eliminate host seed production [Chung et al. 1997]. The benefits from endophytes depend on the fungal and host genotypes and environmental conditions [Cheplick and Faeth 2009].

AGRONOMIC AND ECOLOGICAL IMPLICATIONS

The *Epichloë* symbiosis significantly impacts agricultural practices and natural ecosystems, evidenced by the successful commercialization of novel endophytes, their potential in cereal crops, and the challenges and risks that require careful management and regulation. Asexual, symbiotic *Epichloë* strains are widely used and commercialized in agriculture, most notably in the United States and New Zealand, to reduce livestock toxicity while maintaining pest deterrence in pastures by incorporating selected 'novel endophyte' strains into grass cultivars [Fletcher and Easton 2000, Malinowski et al. 2000]. These selected *Epichloë* strains have become key components of many pasture ecosystems, especially in New Zealand, where an estimated 90% of perennial ryegrass sold contains a selected endophyte strain [Johnson and Caradus 2019, Caradus et al. 2021]. The development and widespread commercialization of "novel endophytes" represent a paradigm shift in agricultural pest management. Instead of relying solely on external chemical applications, the plant itself becomes a "chemical factory," producing its own internal defense mechanisms [Popay and Bonos 2005]. This move towards integrated biological solutions has significant implications for reducing environmental impact and addressing issues like pesticide resistance. The history of *Epichloë*, especially the "novel endophyte" approach, demonstrates a shift toward leveraging internal biological systems. By selecting strains that produce beneficial but non-toxic compounds, agriculture can achieve pest control from within the plant, reducing the need for synthetic chemicals. This approach is much more sustainable and environmentally friendly, aligning perfectly with modern agricultural goals. Moreover, this technology has a significant economic impact; for example, the AR37 endophyte strain alone has contributed approximately NZD 3.6 billion to New Zealand's economy over 20 years [Thom et al. 2012].

BENEFITS OF THE *Epichloë*-GRASS SYMBIOSIS

The symbiosis between *Epichloë* fungi and grasses is an example of a mutually beneficial relationship that offers a number of significant benefits to the host plant and, consequently, to agriculture and the environment. These endophytes, which live within plant tissues without causing disease symptoms, have become the subject of intense research due to their potential as a natural plant protection agent and a factor in improving plant immunity. One key advantage of this symbiosis is its role in biological pest control. *Epichloë* endophytes produce alkaloids that are toxic to herbivorous insects, effectively reducing insect predation [Popay et al. 1995, Popay et al. 2003]. This acts as a built-in, natural defense system for the plant, minimizing the need for chemical pesticides. This not only reduces costs for farmers but also significantly reduces environmental impacts, promoting more sustainable agricultural practices. Furthermore, plants colonized by *Epichloë* exhibit increased resistance to common foliar diseases of grasses and cereals. This includes reduced susceptibility to rust, powdery mildew, and leaf spot, as confirmed by studies [Lee et al. 2021, Card et al. 2021]. This natural defense reduces yield losses due to disease and reduces the need for fungicide intervention. The *Epichloë*-grass symbiosis also contributes to increased plant tolerance to abiotic stresses. Endophytic plants are more resistant to drought, flooding, and potentially other negative effects of climate change [Rodriguez and Redman 2008, Decunta et al. 2021]. The ability to survive in harsh environmental conditions is extremely valuable in the face of increasing climatic challenges and weather variability. Furthermore, the presence of *Epichloë* endophytes is associated with improved vigor, biomass, and seed yield in colonized plants [Malinowski and Belesky 2000]. This translates into healthier and more productive crops, which directly translates into greater agricultural productivity. In the broader context, this symbiosis offers potentially lower costs for farmers and a reduced environmental impact. Reduced demand for chemical pesticides and fertilizers [Johnson et al. 2013] not only lowers farm operating expenses but also supports biodiversity and soil health, contributing to a more ecologically sustainable food system.

CHALLENGES AND RISKS ASSOCIATED WITH *Epichloë* – GRASS SYMBIOSIS

Despite its numerous benefits, the symbiosis between *Epichloë* fungi and grasses also presents certain challenges and potential threats that require attention, especially in the context of their commercial exploitation.

One of the most significant problems is the production of alkaloids by some *Epichloë* strains, which are toxic to livestock. Examples of such substances include ergovaline and lolitrem, which can cause serious conditions such as fescue toxicosis and ryegrass staggers [Dougherty et al. 1991, Bush et al. 1993]. These conditions can lead to significant economic losses in livestock farming, manifesting as decreased weight gain, reduced milk production, reproductive problems, and even death. For this reason, agriculture strives to use grass varieties colonized by *Epichloë* strains which do not produce alkaloids harmful to animals but still provide protection against pests. Another aspect requiring caution is the risk of horizontal fungal transfer when commercially releasing *Epichloë* strains capable of sexual reproduction into the environment. Ascospores produced by these strains can be carried long distances by wind, potentially spreading the fungus to wild grass species or previously uninfected croplands [Glenn et al. 1996]. Such uncontrolled transfer can have unpredictable ecological consequences, including altering the species composition of ecosystems or increasing the stress tolerance of wild grasses, which could affect their invasiveness. Furthermore, the presence of *Epichloë* endophytes in grasses can negatively impact invertebrate biodiversity. Studies have shown that grasses infected with *Epichloë* can reduce populations of beneficial soil organisms, such as earthworms [Omacini et al. 2012]. This is likely due to toxic alkaloids, which can leach into the soil or be consumed by invertebrates feeding on plants or organic matter. Reducing the number of these organisms can disrupt natural ecological processes, such as nutrient cycling and soil aeration. Finally, the increased host plant fitness provided by the symbiosis with *Epichloë* could potentially make grass species invasive in natural or agricultural environments [Clay 1990, Chen et al. 2022]. Plants with *Epichloë* are more competitive, cope better with harsh conditions, and are more resistant to pests and diseases. As a result, they can displace native grass species, disrupting local ecosystems and leading to biodiversity loss. This risk is particularly significant when new *Epichloë* strains are introduced into regions where they do not occur naturally. Understanding these potential threats is crucial for the responsible use of the *Epichloë*-grass symbiosis in agricultural practices and environmental protection.

CONCLUSIONS

Fungi of the genus *Epichloë* are an exceptionally important component of grassland ecosystems and agricultural systems, offering a complex network of interactions that range from mutualistic to antagonistic. Their ability to systematically colonize grasses and produce bioactive alkaloids provides significant benefits to their hosts, such as resistance to pests, diseases, and abiotic stresses, making them valuable tools in sustainable agriculture. Advances in taxonomy, driven by the development of molecular techniques and precise detection methods, have enabled a deeper understanding of their biology and potential.

However, the duality of their action – the ability to produce toxins harmful to livestock (e.g. ergovaline, lolitrem) as well as those that increase resistance to environmental stresses (e.g. peramine) – underscores the need continuous research on this group of fungi and for careful management and stringent regulatory frameworks. The development of "novel endophytes," which provide benefits to plants without harming animals, represents a significant advancement. The future of *Epichloë* research focuses on further harnessing their potential, including expanding their applications to cereal crops and utilizing genetic engineering to tailor alkaloid profiles. These research directions are crucial for addressing global challenges related to food security, climate change, and reducing reliance on chemical plant protection products, while ensuring the responsible and ethical implementation of these powerful biological solutions.

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REFERENCES

Aniszewski, T. (2007). Alkaloids – secrets of life. Alkaloid chemistry, biological significance, aplications and ecological role. Elsevier Radarweg, Amsterdam.

Bastias, D.A., Martínez-Ghersa, M.A., Ballaré, C.L. et al. (2017). *Epichloë* Fungal endophytes and plant defenses: not just alkaloids. Trends Plant Sci., 22(11), 939–948. https://doi.org/10.1016/j.tplants.2017.08.005

Becker, M., Becker, Y., Green, K. et al. (2016). The endophytic symbiont *Epichloë festucae* establishes an epiphyllous net on the surface of *Lolium perenne* leaves by development of an expressorium, an appressorium-like leaf exit structure. New Phytol., 211, 240–25. https://doi.org/10.1111/nph.13931

Berry, D., Mace, W., Grage, K. et al. (2019). Efficient nonenzymatic cyclization and domain shuffling drive pyrrolopyrazine diversity from truncated variants of a fungal NRPS. Proc. Nat. Acad. Sci. (PNAS), 116(51). https://www.pnas.org/cgi/doi/10.1073/pnas.1913080116

Bultman, T.L., White, J.F., Bowdish, T.I. et al. (1998). A new kind of mutualism between fungi and insects. Mycol. Res., 102, 235–238.

Bush, L.P., Fannin, F., Siegel, M. et al. (1993). Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte-grass interactions. Agric. Ecosyst. Environ., 44, 81–102.

Bush, L.P., Wilkinson, H.H, Schardl, C.L. (1997). Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol., 114(1), 1–7. https://doi.org/10.1104/pp.114.1.1

Cagaš, B., Flieger, M., Olsovska, J. (1999). Concentration of ergot alkaloids in Czech ecotypes of *Lolium perenne* and *Festuca pratensis*. Grass Forage Sci., 54, 365–370.

Caradus, J., Chapman, D.F., Cookson, T. et al. (2021). *Epichloë* endophytes – new perspectives on a key ingredient for resilient perennial grass pastures. Resil. Past. Grass. Res. Pract. Ser., 17, 347–360. https://doi.org/10.33584/rps.17.2021.34

Caradus, J.R., Johnson, L.J. (2020). *Epichloë* fungal endophytes – from a biological curiosity in wild grasses to an essential component of resilient high performing ryegrass and fescue pastures. J. Fungi, 6(4), 322. https://doi.org/10.3390/jof6040322

Caradus, J.R. (2023). *Epichloë* fungal endophytes – a vital component for perennial ryegrass survival in New Zealand. New Zeal. J. Agric. Res., 67(4), 451–468. https://doi.org/10.1080/00288233.2023.2170426

Card, S.D., Bastías, D.A., Caradus, J.R. (2021). Antagonism to plant pathogens by *Epichloë* fungal endophytes—a review. Plants, 10(10), 1997.

Charlton, N.D., Craven, K.D., Afkhami, M.E. et al. (2014). Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic *Epichloë* species of *Bromus laevipes*. FEMS Microbiol. Ecol., 90(1), 276–289. https://doi.org/10.1111/1574-6941.12393

Chen, Z., White, J.F., Malik, K. et al. (2022). Soil nutrient dynamics relate to Epichloë endophyte mutualism and nitrogen turnover in a low nitrogen environment. Soil Biol. Biochem., 174, 108832. https://doi.org/10.1016/j.soilbio.2022.108832

Cheplick, G.P., Faeth, S.H. (2009). Ecology and evolution of the grass-endophyte symbiosis. Oxford University Press.

Christensen, M.J. (1995). Variation in the ability of *Acremonium* endophytes of *Lolium perenne*, *Festuca arundinacea* and *F. pratensis* to form compatible associations in the three grasses. Mycol. Res., 99, 466–470. https://doi.org/10.1016/S0953-7562(09)80647-3

Christensen, M.J. (2008). *Epichloë* and *Neotyphodium*: the relationship with the host plant. In: C.L., Schardl, J.F., White, Jr, A.M., de Bary (eds.), The *Epichloë*–grass symbiosis: a new paradigm for mutualism. Springer, 53–73.

Christensen, M.J., Ball, O.J.-P., Bennett, R.J. et al. (1997). Fungal and host genotype effects on compatibility and vascular colonization by *Epichloë festucae*. Mycol. Res., 101, 493–501.

Chung, K.R., Hollin, W., Siegel, M.R. et al. (1997). Genetics of host specificity in *Epichloë typhina*. Phytopathology, 87, 599–605.

Cibils-Stewart, X., Powell, J.R., Popay, A.J. et al. (2020). Reciprocal effects of silicon supply and endophytes on silicon accumulation and *Epichloë* colonization in grasses. Front. Plant Sci., 11, 593198. https://doi.org/10.3389/fpls.2020.593198

Clay, K. (1990). The impact of parasitic and mutualistic fungi on plants. In: J.B., Grace, D. Tilman (eds.), Perspectives on plant competition. Academic Press, San Diego, 391–412.

Clay, K., Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat., 160, Suppl. 4, S99–S127. https://doi.org/10.1086/342161

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology, 69(1), 10–16.

Craven, K.D., Blankenship, J.D., Leuchtmann, A. et al. (2001). Hybrid fungal endophytes symbiotic with the grass *Lolium pratense*. Sydowia 53, 44–73.

Decunta, F.A., Pérez, L.I., Malinowski, D.P. et al. (2021) A systematic review on the effects of *Epichloë* fungal endophytes on drought tolerance in cool-season grasses. Front. Plant Sci., 12, 644731. https://doi.org/10.3389/fpls.2021.644731

Dodds, P., Rathjen, J. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. Nat. Rev. Genet. 11(8), 539–548. https://doi.org/10.1038/nrg2812

Dombrowski, J.E., Baldwin, J.C., Azevedo, M.D. et al. (2006). A sensitive PCR-based assay to detect *Neotyphodium* fungi in seed and plant tissue of tall fescue and ryegrass species. Crop Sci., 46, 1064–1070.

Doss, R.P., Welty, R.E. (1995). A polymerase chain reaction-based procedure for detection of *Acremonium coenophialum* in tall fescue. Phytopathology, 85(8), 913–917.

Dougherty, C.T., Lauriault, L.M., Bradley, N.W. et al. (1991). Induction of tall fescue toxicosis in heat-stressed cattle and its alleviation with thiamin. J. Anim. Sci., 69(3), 1008–1018.

Drake, I., White, Jr, J.F., Belanger, F.C. (2018). Identification of the fungal endophyte of *Ammophila breviligulata* (American beachgrass) as *Epichloë amarillans*. Peer J., 6, e4300. https://doi.org/10.7717/peerj.4300

Du, M., Wang, T., Li, C., Chen, T. (2024). Discovery and characterization of *Epichloë* fungal endophytes from *Elymus* spp. in Northwest China. Microorganisms, 12(7), 1497. https://doi.org/10.3390/microorganisms12071497

Emile, J.C., Surault, F., Bony, S. et al. (2000). Effects of ergovaline in forage grasses on feeding value and animal performance. Proceedings of the 4th International *Neotyphodium*/Grass Interaction Symposium, Soest, Germany, 521–523.

Faeth, S.H., Sullivan, T. (2003). Mutualistic asexual endophytes in a native grass are usually parasitic. Am. Nat., 161(2), 310–325.

Fardella, P. (2024). Harnessing antifungal proteins from fungi to protect plants: a review of the *Epichloë festucae* antifungal protein Efe-AfpA. Grass Res., 4, e003. https://doi.org/10.48130/grares-0024–0001

Fletcher, L.R., Easton, H.S. (2000). Using endophytes for pasture improvement in New Zealand. Proceedings of the 4th International *Neotyphodium*/Grass Interactions Symposium, Soest, Germany, 149–162.

Fredell, G., Bienkowski, D., Light, S.E. et al. (2025). Distribution, potential commercial adoption, and possible impacts of the endophytic fungus, *Epichloë*, in UK pasture grasses and cereals, plant health cases. Plant Health Cases, phcs20250002. https://doi.org/10.1079/planthealthcases.2025.0002

Gams, W., Petrini, O., Schmidt, D. (1990). *Acremonium uncinatum*, a new endophyte in *Festuca pratensis*. Mycotaxon, 37, 67–71.

Grabka, R., d'Entremont, T.W., Adams, S.J. et al. (2022). Fungal endophytes and their role in agricultural plant protection against pests and pathogens. Plants, 11, 384. https://doi.org/10.3390/plants11030384

Guerre, P. (2015). Ergot alkaloids produced by endophytic fungi of the genus *Epichloë*. Toxins, 7(3), 773–790. https://doi.org/10.3390/toxins7030773

Gundel, P.E., Biganzoli, F., Freitas, P.P. et al. (2020). Plant damage, seed production and persistence of the fungal endophyte *Epichloë occultans* in *Lolium multiflorum* plants under an herbivore lepidopteran attack and ozone pollution. Ecol. Austral., 30, 321–330.

Gwinn, K.D., Collins-Shepard, M.H., Reddick, B.B. (1991). Tissue print-immunoblot, an accurate method for the detection of *Acremonium coenophialum* in tall fescue. Phytopathology, 81(7), 747–748.

Hill, N.S., Hiatt, E.E., De Battista, J.P. et al. (2002). Seed testing for endophytes by microscope and immunoblot procedures. Seed Sci. Technol., 30(2), 347–355.

Huggett, J.F., Whale, A. (2013). Digital PCR as a novel technology and its potential implications for molecular diagnostics. Clin. Chem. 59(12), 1691–1693. https://doi.org/10.1373/clinchem.2013.214742

Johnson, L.J., Caradus, J.R. (2019). The science required to deliver *Epichloë* endophytes to commerce. In: T.R., Hodkinson, F.M., Doohan, M.J. et al. (eds.), Endophytes for a growing world. Chapter 16. Cambridge University Press, Cambrige, 343–370. https://doi.org/10.1017/9781108607667.017

Johnson, L.J., de Bonth, A.C.M., Briggs, L.R. et al. (2013). The exploitation of *Epichloë* endophytes for agricultural benefit. Fung. Div., 60, 171–188. https://doi.org/10.1007/s13225-013-0239-4

Jones, J.D.G., Dangl, J.L. (2006). The plant immune system. Nature, 444(7117), 323–329.

Karpyn Esqueda, M., Yen, A.L., Rochfort, S. et al. (2017). A review of perennial ryegrass endophytes and their potential use in the management of African black beetle in perennial grazing systems in Australia. Front. Plant Sci., 8(3). https://doi.org/10.3389/fpls.2017.00003

Kuldau, G., Bacon, C. (2008). *Clavicipitaceous* endophytes: their ability to enhance resistance of grasses to multiple stresses. Biol. Cont., 46(1), 57–71. https://doi.org/10.1016/j.biocontrol.2008.01.023

Latch, G.C.M., Christensen, M.J. (1985). Artificial infection of grasses with endophytes. Ann. Appl. Biol., 107(1), 17–24.

Lee, K., Missaoui, A., Mahmud, K. et al. (2021). Interaction between grasses and *Epichloë* endophytes and its significance to biotic and abiotic stress tolerance and the rhizosphere. Microorganisms, 9(11), 2186. https://doi.org/10.3390/microorganisms9112186

Leuchtmann, A., Schmidt, D., Bush, P.L. (2000). Different levels of protective alkaloids in grasses with stroma-forming and seed-transmited *Epichloë*/*Neotyphodium* endophytes. J. Chem. Ecol., 26(4), 1025–1035.

Leuchtmann, A., Bacon, C.W., Schardl, C.L. et al. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia, 106(2), 202–215. https://doi.org/10.3852/13-251

Li, F., Guo, Y., Christensen, M.J. et al. (2018). An arbuscular mycorrhizal fungus and *Epichloë festucae* var. *lolii* reduce *Bipolaris sorokiniana* disease incidence and improve perennial ryegrass growth. Mycorrhiza, 28(2), 159–169. https://doi.org/10.1007/s00572-017-0813-9

Luna-Fontalvo, J.A., Balocchi, O., Martínez, O. et al. (2025). Symbiosis between *Epichloë* fungi and *Bromus* grasses: a review of current knowledge and future directions. J. Fungi, 11, 807. https://doi.org/10.3390/jof11110807

Ma, J.F., Yamaji, N. (2006). Silicon uptake and accumulation in higher plants. Trends Plant Sci., 11(8), 392–397. https://doi.org/10.1016/j.tplants.2006.06.007

Malinowski, D.P., Alloush, G.A., Belesky, D.P. (2000). Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. Plant Soil, 227, 115–126. https://doi.org/10.1023/A:1026518828237

Malinowski, D.P., Belesky, D.P. (2000). Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci., 40, 923– 940. http://dx.doi.org/10.2135/cropsci2000.404923x

Malinowski, D.P., Belesky, D.P. (2019). *Epichloë* (formerly *Neotyphodium*) fungal endophytes increase adaptation of cool-season perennial grasses to environmental stresses. Acta Agrobot., 72(2), 1767. https://doi.org/10.5586/aa.1767

Mathew, S.A., Helander, M., Saikkonen, K. et al. (2022). *Epichloë* endophytes shape the foliar endophytic fungal microbiome and alter the auxin and salicylic acid phytohormone levels in two meadow fescue cultivars. J. Fungi, 9(1), 90. https://doi.org/10.3390/jof9010090

Moon, C.D., Craven, K.D., Leuchtmann, A. et al. (2004). Prevalence of interspecific hybrids amongst asexual fungal endophytes of grasses. Mol. Ecol. 13(6), 1455–1467. https://doi.org/10.1111/j.1365-294X.2004.02138.x

Moon, C.D., Miles, C.O., Järlfors, U. et al. (2002). The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. Mycologia, 94(4), 694–711.

Moon, C.D., Scott, B., Schardl, C.L. et al. (2000). The evolutionary origins of *Epichloë* endophytes from annual ryegrasses. Mycologia, 92(6), 1103–1118. https://doi.org/10.1080/00275514.2000.12061258

Moore, J.R., Pratley, J.E., Mace et al. (2015). Variation in alkaloid production from genetically diverse *Lolium* accessions infected with *Epichloë* species. J. Agric. Food Chem., 63(48), 10355–10365. https://doi.org/10.1021/acs.jafc.5b03089

Nagabhyru, P., Dinkins, R.D., Schardl, C.L. (2022). Transcriptome analysis of *Epichloë* strains in tall fescue in response to drought stress. Mycologia, 114(4), 697–712. https://doi.org/10.1080/00275514.2022.2060008

Oliveira, J.A., Rottinghaus, G.E., Collar, J. et al. (1997). Perennial ryegrass endophytes in Galicia, Northwest Spain. J. Agric. Sci., 129(2), 173–177. https://doi.org/10.1017/S0021859697004711

Omacini, M., Semmartin, M., Pérez, L.I. et al. (2012). Grass–endophyte symbiosis: a neglected aboveground interaction with multiple belowground consequences. Appl. Soil Ecol., 61, 273–279. https://doi.org/10.1016/j.apsoil.2011.10.012

Philipson, M.N., Christey, M.C. (1986). The relationship of host and endophyte during flowering, seed formation, and germination of *Lolium perenne*. New Zeal. J. Bot., 24(1), 125–134. https://doi.org/10.1080/0028825x.1986.10409724

Popay, A.J., Bonos, S.A. (2005). Biotic responses in endophytic grasses. In: C.A., Roberts, C.P. West, D.E., Spiers (eds.), *Neotyphodium* in cool-season grasses. Blackwell Publishing, Iowa, 163–185.

Popay, A.J., Hume, D.E., Mainland, R.A. et al. (1995). Field resistance to Argentine stem weevil (*Listronotus bonariensis*) in different ryegrasscultivars infected with an endophyte deficient in lolitrem B. New Zeal. J. Agric. Res., 38, 519–528. https://doi.org/10.1080/00288233.1995.9513154

Popay, A.J., Townsend, R.J., Fletcher, L.R. (2003). The effect of endophyte (*Neotyphodium uncinatum*) in meadow fescue on grass grub larvae. New Zeal. Plant Prot., 56, 123–128. https://doi.org/10.1002/9780470384916.ch7

Pozo, M.J., Zabalgogeazcoa, I., Vazquez de Aldana, B.R. et al. (2021). Untapping the potential of plant mycobiomes for applications in agriculture. Curr. Opin. Plant Biol., 60, 102034. https://doi.org/10.1016/j.pbi.2021.102034

Realini, F.M., Escobedo, V.M., Ueno, A.C. et al. (2024). Anti-herbivory defences delivered by Epichloë fungal endophytes: a quantitative review of alkaloid concentration variation among hosts and plant parts. Ann Bot., 133(4), 509–520. https://doi.org/10.1093/aob/mcae014

Redman, R.S., Sheehan, K.B., Stout, R.G. et al. (2002). Thermotolerance generated by plant/fungal symbiosis. Science, 298(5598), 1581. https://doi.org/10.1126/science.1078055

Rodriguez, R.J., Redman, R.S. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J. Exp. Bot., 59(5), 1109–1114. https://doi.org/10.1093/jxb/erm342

Rodriguez, R.J., White Jr, J.F., Arnold, A.E. et al. (2009). Fungal endophytes: diversity and functional roles. New Phytol., 182(2), 314–330. https://doi.org/10.1111/j.1469-8137.2009.02773.x

Rozpądek, P., Wężowicz, K., Nosek, M. et al. (2015). The fungal endophyte *Epichloë typhina* improves photosynthesis efficiency of its host orchard grass (*Dactylis glomerata*). Planta, 242, 1025–1035. https://doi.org/10.1007/s00425-015-2337-x

Rudolph, W., Remane, D., Wissenbach, D.K. et al. (2018). Development and validation of an ultrahigh performance liquid chromatography-high resolution tandem mass spectrometry assay for nine toxic alkaloids from endophyte-infected pasture grasses in horse serum. J. Chromatogr. A, 1560, 35–44. https://doi.org/10.1016/j.chroma.2018.05.013

Saikkonen, K., Young, C.A., Helander, M. et al. (2016). Endophytic *Epichloë* species and their grass hosts: from evolution to applications. Plant Mol. Biol., 90, 665–675. https://doi.org/10.1007/s11103-015-0399-6

Saikkonen, K., Faeth, S.H., Helander, M. et al. (1998). Fungal endophytes: a continuum of interactions with host plants. Ann. Rev. Ecol. Syst., 29, 319–343. https://doi.org/10.1146/annurev.ecolsys.29.1.319

Sarkar, S., Dey, A., Kumar, V. et al. (2021). Fungal endophyte: an interactive endosymbiont with the capability of modulating host physiology in myriad ways. Front. Plant Sci., 12, 701800. https://doi.org/10.3389/fpls.2021.701800

Schardl, C.L. (1996). *Epichloë* species: fungal symbionts of grasses. Ann. Rev. Phytopathol., 34(1), 109–130. https://doi.org/10.1146/annurev.phyto.34.1.109

Schardl, C.L. (2010). The *Epichloë*, symbionts of the grass subfamily *Poöideae*. Ann. Missouri Bot. Gard., 97, 646–665. https://doi.org/10.3417/2009144

Schardl, C.L., Craven, K.D. (2003). Interspecific hybridization in plant-associated fungi and oomycetes: a review. Mol. Ecol., 12(11), 2861–2873. https://doi.org/10.1046/j.1365-294x.2003.01965.x

Schardl, C.L., Leuchtmann, A. (2005). The *Epichloë* endophytes of grasses and the symbiotic continuum. In: J., Dighton, J.F., White, Jr, P.V., Oudemans (eds.), The fungal community. CRC Press, Boca Raton.

Schardl, C.L., Leuchtmann, A., Spiering, M.J. (2004). Symbioses of grasses with seedborne fungal endophytes. Ann. Rev. Plant Biol., 55, 315–340. https://doi.org/10.1146/annurev.arplant.55.031903.141735

Schardl, C.L., Young, C.A., Faulkner, J.R. et al. (2012). Chemotypic diversity of *Epichloë*, fungal symbionts of grasses. Fungal Ecol., 5(3), 331–344. https://doi.org/10.1016/j.funeco.2011.04.005

Schardl, C.L., Florea, S., Pan, J. et al. (2013a). The *Epichloë*: alkaloid diversity and roles in symbiosis with grasses. Curr. Opin. Plant. Biol., 16(4), 480–488. https://doi.org/10.1016/j.pbi.2013.06.012

Schardl, C.L., Young, C.A., Hesse, U. et al. (2013b). Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genet., 9(2), e1003323. https://doi.org/10.1371/journal.pgen.1003323

Schardl, C.L., Florea, S., Nagabhyru, P. et al. (2025). Chemotypic diversity of bioprotective grass endophytes based on genome analyses, with new insights from a Mediterranean-climate region in Isfahan Province, Iran. Mycologia 117(1), 34–59. https://doi.org/10.1080/00275514.2024.2430174

Schreiber, M., Jayakodi, M., Stein, N. et al. (2024). Plant pangenomes for crop improvement, biodiversity and evolution. Nat. Rev. Genet., 25(8), 563–577. https://doi.org/10.1038/s41576-024-00691-4

Scott, B., Becker, Y., Becker, M. et al. (2012). Morphogenesis, growth and development of the grass symbiont *Epichloë festucae*. In: J.P. Martin, A., Di Pietr (eds.). Morphogenesis and pathogenicity in fungi. Springer-Verlag, Heidelberg, 243–264. https://doi.org/10.1007/978-3-642-22916-9_12

Sharma, R., Tangjang, S., Shukla, A.C. (2020). Molecular characterization of endophytic fungi associated with *Centella asiatica* Linn., inhabiting wildly in Arunachal Pradesh. J. Ind. Bot. Soc., 100(3–4), 160–168. https://doi.org/10.5958/2455-7218.2020.00038.8

Siegel, M.R., Bush, L.P. (1996). Defensive chemicals in grass-fungal endophyte associations. Rec. Adv. Phytochem., 30, 81–119. In: J.T., Romeo, J.A., Saunders, P., Barbosa, (eds) phytochemical diversity and redundancy in ecological interactions. Recent Advances in Phytochemistry, vol. 30. Springer, Boston. https://doi.org/10.1007/978-1-4899-1754-6_4

Siegel, M.R., Dahlman, D.L., Bush, L.P. (1989). The role of endophytic fungi in grasses: new approchs to biological control of pests. In: A.R., Leslie, R.L. Metcalf (eds.), Integrated pest management for turfgrass and ornamentals. U.S. Environmental Protection Agency, Washington, D.C., 169–186.

Siegel, M.R., Latch, G.C.M., Johnson, M.C. (1985). *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: significance and control. Plant Dis., 69(2), 179–183.

Stone, J.K., Bacon, C.W., White, J.F. (2000). An overview of endophytic microbes: endophytism defined. In: C.W., Bacon, J.F. White (eds.), Microbial endophytes. Marcel Dekker, New York, 3–29.

Takach, J.E., Mittal, S., Swoboda, G.A. et al. (2012). Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. Appl. Environ. Microbiol., 78(16), 5501–5510. https://doi.org/10.1128/AEM.01084-12

Takach, J.E., Young, C.A. (2014). Alkaloid genotype diversityof tall fescue endophytes. Crop Sci., 54, 667–678. https://doi.org/10.2135/cropsci2013.06.0423

Talamantes, D.R., Phillips, C., Young, C. et al. (2025). The relationships among alkaloid production, fungal biomass, and host genetics in the tall fescue-*Epichloë* symbiosis. bioRxiv preprint. https://doi.org/10.1101/2025.05.30.657052

Thom, E.R., Popay, A.J., Hume, D.E. et al. (2012). Evaluating the performance of endophytes in farm systems to improve farmer outcomes – a review. Crop Past. Sci., 63(10), 927–943, http://dx.doi.org/10.1071/CP12152

Tian, B., Xie, J., Fu, Y. et al. (2020). A cosmopolitan fungal pathogen of dicots adopts an endophytic lifestyle on cereal crops and protects them from major fungal diseases. ISME J., 14(12), 3120–3135. https://doi.org/10.1038/s41396-020-00744-6

Tsai, H.F., Liu, J.S., Staben, C. et al. (1994). Evolutionary diversification of fungal endophytes of tall fescue by hybridization with *Epichloë* species. Proc. Nat. Acad. Sci., 91(7), 2542–2546. https://doi.org/10.1073/pnas.91.7.2542

Vassiliadis, S., Elkins, A.C., Reddy, P. et al. (2019). A simple LC–MS method for the quantitation of alkaloids in endophyte-infected perennial ryegrass. Toxins, 11, 649. https://doi.org/10.3390/toxins11110649

Vassiliadis, S., Reddy, P., Hemsworth, J. et al. (2023). Quantitation and distribution of *Epichloë*-derived alkaloids in perennial ryegrass tissues. Metabolites, 13, 205. https://doi.org/10.3390/metabo13020205

Vazquez de Aldana, B.R., Garcia Ciudad, A., Zabalgogeazcoa, I. et al. (2001). Ergovaline levels in cultivars of *Festuca arundinacea*. Animal. Feed Sci. Technol., 93, 169–176. https://doi.org/10.1016/S0377-8401(01)00285-1

Vikuk, V., Young, C.A., Lee, S.T. et al. (2019). Infection rates and alkaloid patterns of different grass species with systemic Epichloë endophytes. Appl. Environ. Microbiol., 85(17), e00465-19. https://doi.org/10.1128/AEM.00465-19

von Cräutlein, M., Helander, M., Korpelainen, H. et al. (2021). Genetic diversity of the symbiotic fungus *Epichloë festucae* in naturally occurring host grass populations. Front. Microbiol., 12, 756991. https://doi.org/10.3389/fmicb.2021.756991

Wang, C., Shi, C., Huang, W. et al. (2024). The impact of aboveground *Epichloë* endophytic fungi on the rhizosphere microbial functions of the host *Melica transsilvanica*. Microorganisms, 12, 956, https://doi.org/10.3390/microorganisms12050956

Wang, J., Hou, W., Christensen, M.J. et al. (2020). Role of *Epichloë* endophytes in improving host grass resistance ability and soil properties. J. Agric. Food Chem., 68(26), 6944–6955. https://doi.org/10.1021/acs.jafc.0c01396

Wang, Z., Zhao, F., Bao, Q., Liu, X., Guo, C. (2025). Identification and characterization of three *Epichloë* endophytes isolated from wild barley in China. J Fungi., 11(2), 142. https://doi.org/https://doi.org/10.3390/jof11020142

White Jr, J.F. (1994). Taxonomic relationships among the members of the *Balansiae* (*Clavicipitales*). In: C.W., Bacon, J.F., White, Jr (eds.), Biotechnology of endophytic fungi of grasses. CRC Press, Boca Raton, 3–20.

White Jr, J.F. (1997). Systematic of the graminicolous *Clavicipitaceae*: applications of morphological and molecular approaches. In: C.W., Bacon, N.S., Hill (eds.), *Neotyphodium*/grass interactions. Plenum Press, New York, 27–39.

White Jr, J.F., Cole, G.T. (1985). Endophyte – host associations in forage grasses. I. Distributions of fungal endophytes in some species of *Lolium* and *Festuca*. Mycologia, 77, 323–327.

White Jr, J.F., Reddy, P.V. (1998). Examination of structure and molecular phylogenetic relationships of some Graminicolous symbionts in genera *Epichloë* and *Parepichloë*. Mycologia, 90, 226–234.

Wiewióra, B. (2011). Grzyby endofityczne z rodzaju *Neotyphodium* występujące w trawach wieloletnich w Polsce oraz ich znaczenie dla upraw pastewnych i trawnikowych. Mon. Rozpr. Nauk. IHAR-PIB, 38, 1–115.

Wiewióra, B., Żurek, G., Pańka, D. (2015). Is the vertical transmission of *Neotyphodium lolii* in perennial ryegrass the only possible way to the spread of endophytes? Plos One, 10(2), e 0117231. https://doi.org/10.1371/journal.pone.0117231

Wilson, D. (1995). Endophyte: the evolution of a term and clarification of its use and definition. Oikos, 73, 274–276. http://dx.doi.org/10.2307/3545919

Zabalgogeazcoa, I. (2008). Review. Fungal endophytes and their interaction with plant pathogens. Spanish J. Agric. Res., 6 (special issue), 138–146.

Zhao, X., Muhammad Aslam, M., Chen, M. et al. (2025). Plant–fungi mutualism, alternative splicing, and defense responses: balancing symbiosis and immunity. Int. J. Mol. Sci., 26, 5197. https://doi.org/10.3390/ijms26115197

Zhong, R., Zhang, L., Zhang, X. (2022). Allelopathic effects of foliar *Epichloë* endophytes on belowground arbuscular mycorrhizal fungi: a meta-analysis. Agriculture, 12, 1768. https://doi.org/10.3390/ agriculture12111768

Żurek, M., Ochodzki, P., Wiewióra, B. (2010). Ocena zawartości ergowaliny w trawach runi wybranych trwałych użytków zielonych na terenie województwa mazowieckiego. Biul. IHAR, 257–258, 39–47. https://doi.org/10.37317/biul-2010-0004

CONTENTS