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NONDESTRUCTIVE DISCRIMINATION OF ADVANCED CLONES AND CULTIVARS OF STRAWBERRY USING AN INNOVATIVE APPROACH INVOLVING IMAGE ANALYSIS AND MACHINE LEARNING

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ABSTRACT

Different clones and cultivars of strawberry can differ in morphological and chemical properties, as well as productivity, adaptation to cultivation conditions, and post-harvest quality during storage and processing. Due to differences in the quality of raw materials and final products depending on the strawberry clone/cultivar, correct distinguishing clones and cultivars is important for growers, consumers and processors. This study was aimed at distinguishing advanced clones and cultivars of strawberry using an innovative approach involving image processing and artificial intelligence. The raw material included the advanced clones and cultivars of strawberry, such as clone with the breeding code T-201457-16 (Grandarosa × Elsanta), clone T-201536-06 (Clery × Grandarosa), clone T-201567-01 (Patty × Panvik), as well as the cultivars Fibion, Grandarosa, and Markat. The fruit image acquisition was performed using a digital camera. As many as 2172 image parameters were extracted from the image of each fruit converted to different color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S* and textures with the highest discriminative power were selected to develop models using various machine learning algorithms, such as Multilayer Perceptron, MultiClass Classifier, IBk, and LMT, Linear Discriminant, Quadratic SVM, Subspace Discriminant, and Wide Neural Network. The most accurate classifications were obtained for a model built using Subspace Discriminant (96.30%) and Multilayer Perceptron (95.83%). For the model developed using Subspace Discriminant, clone T-201567-01 and cultivar Markat were completely correctly classified with the highest accuracy of 100%. Whereas in the case of the model built using Multilayer Perceptron clone T-201567-01 was characterized by the highest classification metrics, such as Precision and F-measure equal to 0.983, MCC of 0.980, PRC Area and ROC Area of 1.000. The developed approach can be used in practice to discriminate advanced clones and cultivars of strawberry in an objective and nondestructive manner.

Keywords: image textures, classification models, fruit, Subspace Discriminant, Multilayer Perceptron

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) is a herbaceous plant cultivated and consumed worldwide [Sun et al. 2023, Şener et al. 2023]. Strawberries are planted due to their red, aromatic, and sweet fruit [Patel et al. 2023]. The red color and unique flavor resulting from the combination of taste, aroma, and mouthfeel sensations especially attract the consumers. Thus, strawberries can be intended for the fresh market or

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used as food ingredients in bakery and dairy products, as well as in beverages [Teribia et al. 2021]. Strawberry can be considered as a functional food source with benefits to human health, since it's rich with numerous nutritional compounds such as polyphenols, antioxidants, vitamins, fiber, minerals, and trace elements. Due to the presence of these compounds, strawberry is characterized by antioxidant, anti-aging, pro-digestive, anti-inflammatory, antihypertensive, antiproliferative, and antihyperlipidemic activities [Şener et al. 2023, Ladika et al. 2024, Tang et al. 2024].

The color, shape and chemical properties of strawberries can differ depending on the cultivar [Sturm et al. 2003, Boonyakiat et al. 2016, Parra-Palma et al. 2020, Lee et al. 2022]. Furthermore, strawberry can be characterized by different volatile and taste profiles with specific and balanced stability during processing depending on the cultivar. For this reason, final products belonging to different cultivars are qualitatively different after processing and behave dissimilarly during storage, leading food manufacturers to pay more attention to raw material selection [Teribia et al. 2021].

Moreover, many strawberry breeding programs were carried out to develop new cultivars or improved clones better adapted to environmental conditions, more productive, with higher disease resistance and with better post-harvest quality [Galvão et al. 2017]. The resilience and adaptation to cultivation conditions can also depend on cultivars and clones. Therefore, clone selection with improved resistance is with importance in strawberry stress tolerance breeding [Dziadczyk et al. 2003]. Additionally, there may be differences in morphological characteristics in terms of color, brightness, shape, and size of individual clones and cultivars [de Souzam et al. 2021]. The distinguishing or identification of cultivars is essential to protect the breeder's rights, select improved cultivars for breeding programs and meet consumer needs. Initially, the strawberry cultivar identification was performed using morphological features and then biochemical markers such as isozymes [Jung et al. 2017]. For the effective assessment of strawberry genetic diversity and identification of cultivars and clones, molecular markers can also be applied [Tyrka et al. 2002, Whitaker 2011, Jung et al. 2017]. Both morphological and molecular analyses are destructive and require tedious and time-consuming work as well as hard technicity to achieve cultival discrimination. Therefore, the added value of artificial intelligence technologies can be explored.

Thus, the objective of this study was to distinguish advanced clones and cultivars of strawberry using a nondestructive, objective, and inexpensive approach involving image analysis and artificial intelligence. The innovative models were built based on selected textures extracted from images in different color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S*.

MATERIALS AND METHODS

Materials

The research included fruit of advanced clones and cultivars of strawberry (Tab. 1), collected during the fully-ripening period of the plants in a field trial of the National Institute of Horticultural Research in Skierniewice, Poland. Each genotype was represented by 60 plants, grown in the soil and managed in accordance with recommendations for commercial plantations (mechanical and manual removal of weeds and runners, plant irrigation using self-propelled sprinkler, fertilization with YaraMila™ Complex multi-component fertilizer, integrated protection against diseases and pests in accordance with the current Strawberry Plant Protection Program). All the tested genotypes were characterized by very high productivity, large, attractive and firm fruits as well as low plant susceptibility to fungal leaf diseases. Fruits selected for the studies were uniform in shape and color, typical for

Table 1. Clones and cultivars of strawberry used in the experiment

Clones (breeding code and pedigree)	Cultivars
T-201536-06, pedigree Clery × Grandarosa	Fibion
T-201567-01, pedigree Patty × Panvik	Grandarosa
T-201457-16, pedigree Grandarosa × Elsanta	Markat

each of the genotype. Strawberries were washed and cleaned directly after harvesting, and then subjected to image acquisition.

Image acquisition and processing

The strawberry images were acquired using a digital camera (Canon Inc., Tokyo, Japan) on a black background using light-emitting diode illumination. Fruit images were obtained in one hundred repetitions for each clone of T-201536-06, T-201567-01, and T-201457-16, and each cultivar of Fibion, Grandarosa, and Markat. The acquired images were processed using MaZda software (Łódź University of Technology, Institute of Electronics, Łódź, Poland) [Szczypiński et al. 2007, Szczypiński et al. 2009, Strzelecki et al. 2013]. The image processing included image conversion to color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S*, image segmentation based on the intensity of pixel brightness, and ROI (region of interest) determination. The last step was the texture extraction from images. For each fruit considered as one ROI, 2172 image texture parameters were computed based on the run-length matrix, co-occurrence matrix, autoregressive model, histogram, Haar wavelet transform, and gradient map.

Distinguishing advanced clones and cultivars of strawberry using machine learning models

Strawberry advanced clones and cultivars, such as T-201536-06, T-201567-01, T-201457-16, Fibion, Grandarosa, Markat were distinguished using models built based on selected image texture parameters. Various models were developed using WEKA machine learning software (Machine Learning Group, University of Waikato, Hamilton, New Zealand) [Witten and Frank 2005, Bouckaert et al. 2016, Frank et al. 2016] and MATLAB (MathWorks, Inc., Natick, MA, USA). Before building classification models, image textures with the highest discriminative power were selected by Best First using WEKA. The same set of selected textures was used to build models using both WEKA and MATLAB. A test mode of 10-fold cross-validation was applied in both cases. For machine learning models developed using WEKA, different algorithms from groups of Functions, Bayes, Meta, Lazy, Trees, and Rules were used. It was observed that the classifiers providing the highest correctness were Multilayer Perceptron from Functions, MultiClass Classifier from Meta, IBk from Lazy, and LMT from Trees. The parameters of classifiers used to build models are presented in Table 2.

For a model developed using each classifier, confusion matrix with accuracies for each strawberry class, average accuracy, Kappa statistic, Precision, Recall, MCC (Matthews Correlation Coefficient), F-measure, PRC Area (Precision-Recall Area), and ROC Area (Receiver Operating Characteristic Area) were determined using the Equations 1–10 [Ropelewska 2022, Ropelewska et al. 2022, Unlersen et al. 2022, Ropelewska et al. 2023]. Additionally, the PRC curves and ROC curves for each clone and cultivar were determined in the case of the model characterized by the highest average accuracy.

Table 2. The model parameters of classifiers applied to distinguish strawberry advanced clones and cultivars using WEKA

Classifier	Parameters
Multilayer Perceptron	autoBuild: True; batchSize: 100; decay: False; debug: False; doNotCheckCapabilities: False; momentum: 0.2; learningRate: 0.3; hiddenLayers: a; nominalToBinaryFilter: True; validationTreshold: 20; normalizeNumericClass: True; normalizeAttributes: True; reset: True; trainingTime: 500; resume: False; seed: 0
MultiClass Classifier	batchSize: 100; classifier: Logistic, ridge: 1.0E–8, maxIts: –1, numDecimalPlaces: 4; debug: False; logLossDecoding: False; doNotCheckCapabilities: False; method: one-against-all; randomWidthFactor: 2.0; seed: 1; use PairwiseCoupling: False
IBk	batchSize: 100; KNN: 1; debug: False; distanceWeighting: No distance weighting; doNotCheckCapabilities: False; meanSquared: False; nearestNeighbourSearchAlgorithm: LinearNNSearch
LMT	batchSize: 100; debug: False; fastRegression: True; doNotCheckCapabilities: False; errorOnProbabilities: False; minNumInstances: 15; numBoostingIterations: –1; splitOnResiduals: False

$$\text{Accuracy} = \frac{(TP + TN)}{TP + TN + FN + FP} \quad (1)$$

$$\text{Kappa} = \frac{\frac{(TP + FP)(TP + FN)}{(TP + FP)(TP + FN)(TN + FP)(TN + FN)} + \frac{(TN + FP)(TN + FN)}{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}{(TP + FP)(TP + FN)(TN + FP)(TN + FN)} \quad (2)$$

$$\text{Precision} = \frac{TP}{TP + FP} \quad (3)$$

$$\text{Recall} = \frac{TP}{TP + FN} \quad (4)$$

$$\text{MCC} = \frac{(TP \cdot TN - FP \cdot FN)}{\sqrt{((TP + FP)(TP + FN)(TN + FP)(TN + FN))}} \quad (5)$$

$$\text{F-measure} = \frac{2 \cdot \text{Precsion} \cdot \text{Recall}}{(\text{Precision} + \text{Recall})} \quad (6)$$

$$\text{PRC Area} = \text{area under Precision vs. Recall curve} \quad (7)$$

$$\text{TPR} = \frac{TP}{TP + FN} \quad (8)$$

$$\text{FPR} = \frac{FP}{FP + TN} \quad (9)$$

$$\text{ROC Area} = \text{area under TPR vs. FPR curve} \quad (10)$$

where TP is true positive; TN is true negative; FP is false positive; FN is false negative; TPR is true positive rate; FPR is false positive rate.

In the case of models developed using MATLAB, also four most effective models were selected. The hy-perparameters of classifiers are presented in Table 3. For the evaluation of classification, the confusion matrices and average accuracies were determined.

RESULTS

Results generated using WEKA software. The confusion matrices and average accuracies of the classifica-

Table 3. The hyperparameters of classifiers used to distinguish strawberry advanced clones and cultivars using MATLAB

Classifier type	Model Hyperparameters
Linear Discriminant	Preset: Linear Discriminant; Covariance structure: Full
SVM	Preset: Quadratic SVM; Model function: Quadratic; Kernel scale: Automatic; Box constraint level: 1; Multiclass method: One-vs-one; Standardize data: Yes
Ensemble	Preset: Subspace Discriminant; Ensemble method: Subspace; Learner type: Discriminant; Number of learners: 30; Subspace dimension: 42
Neural Network	Preset: Wide Neural Network; Number of fully connected layers: 1; Iteration limit: 1000; First layer size: 100; Activation: ReLU; Regularization strength (Lambda): 0; Standardize data: Yes

tion of strawberry advanced clones, such as T-201536-06, T-201567-01, T-201457-16, and cultivars Fibion, Grandarosa, Markat built based on selected texture parameters from images in color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S* using machine learning models are presented in Table 4. The most correct classification was obtained for a model developed using Multilayer Perceptron. The average accuracy reached 95.83%. Also, the Kappa statistic of 0.9500 was the highest for a model built using Multilayer Perceptron. In the case of other algorithms, the average accuracies and values of Kappa statistic were 93.89% and 0.9267 for LMT, 92.78% and 0.9133 for MultiClass Classifier, and 92.22% and 0.9067 for IBk, respectively. In the case of all models, the clone T-201567-01 was characterized by very high classification accuracy, reaching 100% for a model built using LMT, 98% for models developed using Multilayer Perceptron and IBk, and 97% in the case of a model built using MultiClass Classifier. Generally, the highest number of misclassified cases occurred between the clone T-201536-06 and Grandarosa. The high misclassification was also observed between clones T-201536-06 and T-201457-16. The number of cases belonging to Grandarosa and misclassified as T-201536-06 reached 15 for a model built using IBk. For the same model, as many as 7 cases from the actual class T-201536-06 were incorrectly classified as Grandarosa. The highest number of cases belonging to T-201457-16, which were incorrectly included in the predicted class T-201536-06 were equal to 7 and it was observed for a model developed using MultiClass Classifier.

The other classification performance metrics are shown in Table 5. It was found that strawberry clone

Table 4. The accuracies of classification of strawberry advanced clones and cultivars using machine learning models built based on selected image texture parameters

Algorithm	Predicted class (%)						Actual class	Average accuracy (%)
	T-201536-06	T-201567-01	T-201457-16	Fibion	Grandarosa	Markat		
Multilayer Perceptron	93	0	2	2	2	1	T-201536-06	95.83
	0	98	0	0	0	2	T-201567-01	
	2	0	98	0	0	0	T-201457-16	
	0	0	2	95	3	0	Fibion	
	3	2	0	3	92	0	Grandarosa	
	0	0	0	2	0	98	Markat	
MultiClass Classifier	93	2	2	3	0	0	T-201536-06	92.78
	1	97	0	0	0	2	T-201567-01	
	7	0	93	0	0	0	T-201457-16	
	3	0	2	95	0	0	Fibion	
	5	0	0	3	90	2	Grandarosa	
	5	3	0	2	2	88	Markat	
IBk	87	1	3	2	7	0	T-201536-06	92.22
	0	98	0	0	0	2	T-201567-01	
	3	2	93	0	2	0	T-201457-16	
	2	0	1	95	2	0	Fibion	
	15	0	0	2	82	1	Grandarosa	
	0	2	0	0	0	98	Markat	
LMT	88	1	2	2	5	2	T-201536-06	93.89
	0	100	0	0	0	0	T-201567-01	
	3	2	95	0	0	0	T-201457-16	
	2	0	0	95	1	2	Fibion	
	3	2	0	3	92	0	Grandarosa	
	0	0	0	5	2	93	Markat	

Table 5. The performance metrics of distinguishing strawberry advanced clones and cultivars based on selected texture parameters of images

Algorithm	Class	Precision	Recall	MCC	F-measure	PRC Area	ROC Area
Multilayer Perceptron	T-201536-06	0.949	0.933	0.930	0.941	0.977	0.983
	T-201567-01	0.983	0.983	0.980	0.983	1.000	1.000
	T-201457-16	0.967	0.983	0.970	0.975	0.991	0.998
	Fibion	0.934	0.950	0.931	0.942	0.977	0.989
	Grandarosa	0.948	0.917	0.919	0.932	0.994	0.999
	Markat	0.967	0.983	0.970	0.975	0.998	1.000
	Weighted average	0.958	0.958	0.950	0.958	0.989	0.995
MultiClass Classifier	T-201536-06	0.812	0.933	0.843	0.868	0.894	0.966
	T-201567-01	0.951	0.967	0.950	0.959	0.986	0.995
	T-201457-16	0.966	0.933	0.939	0.949	0.972	0.990
	Fibion	0.919	0.950	0.921	0.934	0.979	0.996
	Grandarosa	0.982	0.900	0.929	0.939	0.976	0.993
	Markat	0.964	0.883	0.908	0.922	0.983	0.997
	Weighted average	0.932	0.928	0.915	0.929	0.965	0.989
IBk	T-201536-06	0.813	0.867	0.806	0.839	0.704	0.911
	T-201567-01	0.952	0.983	0.961	0.967	0.929	0.978
	T-201457-16	0.949	0.933	0.930	0.941	0.901	0.957
	Fibion	0.966	0.950	0.950	0.958	0.915	0.964
	Grandarosa	0.891	0.817	0.825	0.852	0.758	0.858
	Markat	0.967	0.983	0.970	0.975	0.932	0.978
	Weighted average	0.923	0.922	0.907	0.922	0.857	0.941
LMT	T-201536-06	0.914	0.883	0.879	0.898	0.967	0.991
	T-201567-01	0.952	1.000	0.971	0.976	0.995	0.999
	T-201457-16	0.983	0.950	0.960	0.966	0.994	0.999
	Fibion	0.905	0.950	0.912	0.927	0.990	0.998
	Grandarosa	0.917	0.917	0.900	0.917	0.972	0.994
	Markat	0.966	0.933	0.939	0.949	0.995	0.999
	Weighted average	0.939	0.939	0.927	0.939	0.986	0.997

MCC – Matthews Correlation Coefficient, PRC Area – Precision-Recall Area, ROC Area – Receiver Operating Characteristic Area

T-201567-01 was distinguished by the highest values of Precision of 0.983, MCC of 0.980, F-measure of 0.983, PRC Area of 1.000, and ROC Area of 1.000 for a model developed using Multilayer Perceptron and the highest Recall of 1.000 for a model built using LMT. These values indicated high classification correctness. The lowest values of Precision of 0.812 (MultiClass Classifier), MCC of 0.806, F-measure of 0.839, and PRC Area of 0.704 (IBk) were determined for T-201536-06. Whereas the lowest Recall of 0.817 and ROC Area of 0.858 (IBk) were obtained for Grandarosa. It indicated low classification accuracies.

In addition to numeric values of classification performance metrics, the PRC (Precision–Recall) curves and ROC curves were determined for the model built using Multilayer Perceptron, which provided the highest average accuracy. The PRC curves for each clone and cultivar are presented in Figure 1 and the ROC curves are

shown in Figure 2. The graphs confirmed very high correctness of distinguishing the clone T-201567-01. Both PRC Area and ROC Area were equal to 1.000, which is visible in Figures 1b and 2b, respectively. Whereas curves presented in Figures 1a and 1d indicate the lowest PRC Area of 0.977 for T-201536-06 and Fibion. The lowest ROC Area of 0.983 for T-201536-06 is confirmed by curves in Figure 1a.

Results generated using MATLAB software

Average accuracies of the classification of strawberry advanced clones and cultivars performed using

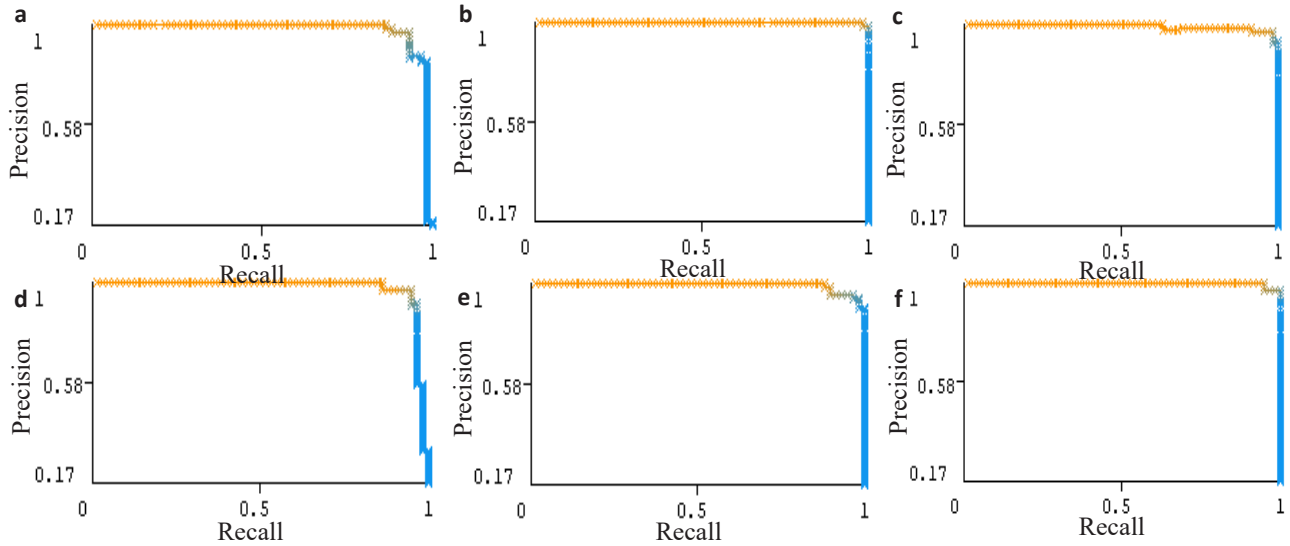

Fig. 1. The PRC (Precision–Recall) curves for distinguishing strawberry advanced clones T-201536-06 (a), T-201567-01 (b), T-201457-16 (c), and cultivars Fibion (d), Grandarosa (e), Markat (f) using Multilayer Perceptron

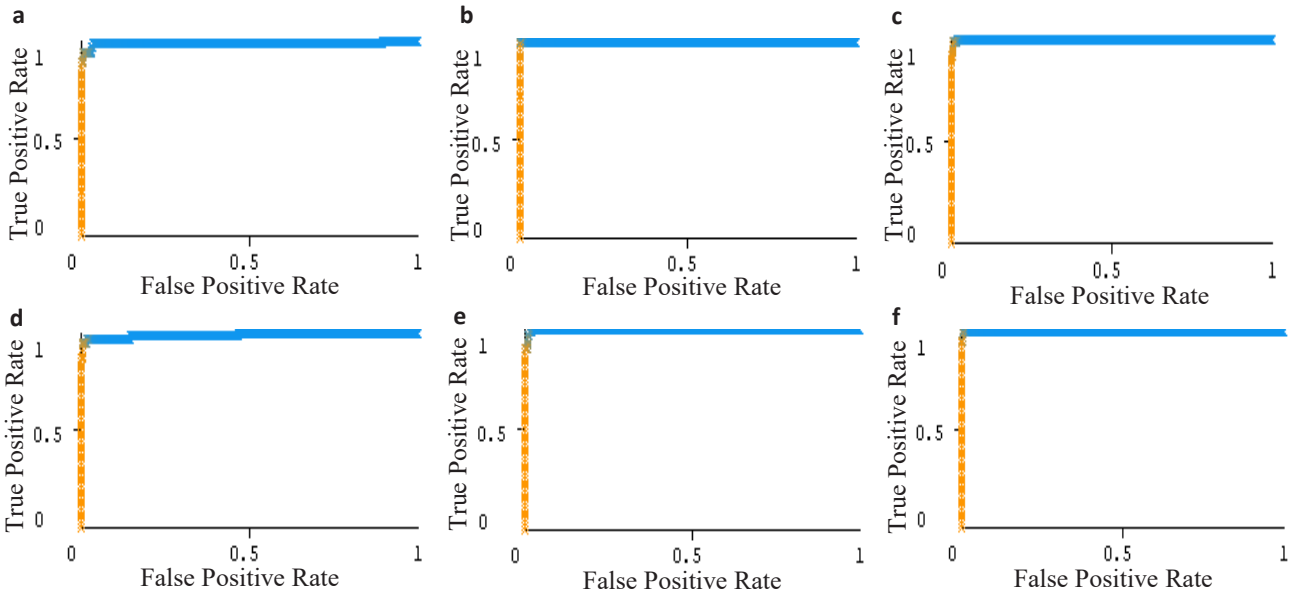

Fig. 2. The ROC (Receiver Operating Characteristic) curves for the classification of strawberry advanced clones T-201536-06 (a), T-201567-01 (b), T-201457-16 (c), and cultivars Fibion (d), Grandarosa (e), Markat (f) using Multilayer Perceptron

a

Predicted class (%)

T-201536-06	T-201567-01	T-201457-16	Fibion	Grandarosa	Markat	Actual class
93	0	0	2	5	0	T-201536-06
0	98	0	0	0	2	T-201567-01
5	0	95	0	0	0	T-201457-16
0	0	1	97	2	0	Fibion
3	0	0	5	92	0	Grandarosa
0	0	0	0	0	100	Markat

b

Predicted class (%)

T-201536-06	T-201567-01	T-201457-16	Fibion	Grandarosa	Markat	Actual class
88	0	3	2	7	0	T-201536-06
0	100	0	0	0	0	T-201567-01
7	0	93	0	0	0	T-201457-16
0	0	1	97	2	0	Fibion
2	0	0	1	97	0	Grandarosa
1	0	0	2	0	97	Markat

c

Predicted class (%)

T-201536-06	T-201567-01	T-201457-16	Fibion	Grandarosa	Markat	Actual class
95	0	2	1	2	0	T-201536-06
0	100	0	0	0	0	T-201567-01
2	0	98	0	0	0	T-201457-16
0	0	2	93	5	0	Fibion
2	1	2	3	92	0	Grandarosa
0	0	0	0	0	100	Markat

d

Predicted class (%)

T-201536-06	T-201567-01	T-201457-16	Fibion	Grandarosa	Markat	Actual class
90	0	5	0	3	2	T-201536-06
0	100	0	0	0	0	T-201567-01
5	0	95	0	0	0	T-201457-16
0	0	1	97	2	0	Fibion
5	1	0	2	92	0	Grandarosa
2	0	0	0	0	98	Markat

Fig. 3. The confusion matrices of classification of strawberry advanced clones and cultivars using models built based on selected image texture parameters using Linear Discriminant (a), Quadratic SVM (b), Sub-space Discriminant (c), and Wide Neural Network (d)

MATLAB were equal to 95.80% for Linear Discriminant, 95.30% for Quadratic SVM, 96.30% for Subspace Discriminant, and 95.30% for Wide Neural Network. The confusion matrices in Figure 3 present the highest classification accuracy of 100.00% for T-201567-01 for three out of four applied models built using Quadratic SVM (Fig. 3b), Subspace Discriminant (Fig. 3c), and Wide Neural Network (Fig. 3d). The lowest accuracy of 88.00% was determined for T-201536-06 in the case of a model built using Quadratic SVM (Fig. 3b). The high number of misclassified cases were between T-201536-06 and Grandarosa and between T-201536-06 and T-201457-16. These observations were similar to the classification results obtained using WEKA (Tab. 4).

DISCUSSION

The approach involving image analysis and artificial intelligence innovative proved to be useful for the discrimination of advanced clones T-201536-06, T-201567-01, and T-201457-16, and cultivars Fibion, Grandarosa, and Markat of strawberry. Models built based on selected texture parameters from images in color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S* using machine learning algorithms were successful and provided a high average accuracy of up to 96.30% for the model developed using MATLAB (Subspace Discriminant) and 95.83% for the model built by WEKA algorithm (Multilayer Perceptron). It showed that the obtained results were very similar, regardless of the applied software. The high discrimination results revealed the great usefulness of image texture parameters for distinguishing strawberry clones and cultivars in a nondestructive, objective, and effective manner.

In the case of models developed using WEKA and MATLAB, the high misclassification of cases was observed between the clone T-201536-06 and Grandarosa and between clones T-201536-06 and T-201457-16. It may be due to the fact that both clones had the Grandarosa cultivar in their pedigree. Generally, for the models built using WEKA and MATLAB, the clone T-201567-01 was discriminated with very high accuracy. It was confirmed by other performance metrics and graphs presented PRC (Precision–Recall) curves and ROC (Receiver Operating Characteristic) curves. It meant that the clone T-201567-01 was very different in terms of image textures from other clones and cultivars.

In the previous literature, there are also reports concerning the application of imaging and artificial intelligence for studies of strawberry. Yamamoto et al. [2015] used the image analysis system for the strawberry quality evaluation and cultivar identification based on appearance characteristics. The classification models built using linear discriminant analysis (LDA) based on a single feature type such as shape, size, or color classified 14 strawberry cultivars with an accuracy of less than 42%. However, the accuracy increased to 68% for a model combining shape, size, and color features. Nevertheless, the obtained accuracy of 68% was lower than the accuracies determined in our study for models developed based on image textures. Whereas Amoriello et al. [2022] predicted the internal quality features of strawberry based on color parameters, such as *L**, *a**, and *b** using the artificial neural network (ANN) and multiple linear regression models (MLR). The application of ANN allowed for obtaining high prediction results, such as $R^2 = 0.906$, and $R^2 = 0.943$ for antioxidant activity and the total monomeric anthocyanin, respectively. Color images and neural networks were also used by Choi et al. [2021] for the evaluation of the strawberry external quality. The recognition models developed based on RGB images using convolutional neural networks (CNNs) allowed for the distinguishing fresh, moldy, and bruised strawberries with the correctness reaching 97%. Computer vision combined with deep learning was also used by Patel et al. [2021] to detect the strawberry plant wetness.

In addition to color images, also hyperspectral imaging combined with artificial intelligence was used for strawberry quality evaluation. For example, real-time hyperspectral imaging and deep learning were applied for the in-field strawberry ripeness estimation providing a classification accuracy of 98.6% for the early ripe and ripe samples [Gao et al. 2020]. Hyperspectral imaging combined with deep learning was also used for strawberry maturity determination and soluble solids content estimation [Su et al. 2021], combined with support vector machine (SVM) for strawberry ripeness evaluation [Zhang et al. 2016], and with SVM and back propagation neural network (BPNN) for the identification of healthy, and bruise and fungi infected strawberries [Liu et al. 2018].

The above-mentioned literature data confirmed the usefulness of imaging and artificial intelligence for the determination of external and internal quality of strawberries and for strawberry classification. Our study expanded knowledge about the application of color imaging and machine learning and set new directions in nondestructive strawberry quality evaluation. It was revealed that image texture parameters can be useful for distinguishing strawberries based on external appearance. Undertaken studies can be continued by involving more clones and cultivars, internal features, and deep learning. The approach combining image analysis and artificial intelligence can be useful in practice to distinguish clones and cultivars of strawberry in a nondestructive and objective manner.

CONCLUSIONS

In this study, an objective, nondestructive, and inexpensive approach involving image analysis and artificial intelligence was developed to classify advanced clones and cultivars of strawberry. The innovative models developed based on selected textures from images in color channels *R*, *G*, *B*, *L*, *a*, *b*, *X*, *Y*, *Z*, *U*, *V*, and *S* proved to be useful for distinguishing advanced clones T-201536-06, T-201567-01, and T-201457-16, and cultivars Fibion, Grandarosa, and Markat with an average accuracy reaching 95.83% and 96.30% for models built using Multilayer Perceptron and Subspace Discriminant, respectively. The applied approach is a novelty in strawberry clone and cultivar classification. The procedure is characterized by great practical applications. Including image textures selected from a set of as many as 2172 parameters in the classification models allowed for very accurate discrimination of advanced clones and cultivar of strawberry. This approach based on image analysis and artificial intelligence may be useful for strawberry growers and processors. However, further research can be carried out, involving a larger number of clones and cultivars, fruit internal features, and deep learning.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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EFFECTIVENESS OF UAN FERTILISATION WITH POTASSIUM THIOSULPHATE IN PEPPER AND TOMATO CULTIVATION

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ABSTRACT

The European Commission proposed the European Green Deal, aiming to reduce plant nutrient losses by at least 50% while preventing soil fertility deterioration and reducing fertiliser use by at least 20% by 2030. Of particular importance for environmental reasons is the reduction of nitrogen fertilisation rates. UAN is a highly concentrated nitrogen fertiliser in an aqueous solution of nitrate and urea ammonium nitrate. This study evaluated the effectiveness of fertilisation in pepper and tomato cultivation using UAN mixtures with potassi um thiosulphate in proportions selected based on a model pot experiment. The field experiment was conducted from 2019 to 2020 at the Felin Experimental Farm of the University of Life Sciences in Lublin. The test plants were sweet peppers of the Balta F_1 cultivar *(Capsicum annuum* L.) and tomatoes of the Mirsini cultivar (*Lycopersicon esculentum* Mill.). The experiment included the following variable factors: nitrogen dose (2 levels: N_1 – optimum nitrogen rate and N_2 – nitrogen rate reduced by 25% from the optimum dose) and fertiliser composition (2 levels: pure UAN – $N : K_0 : S_0$, UAN with potassium thiosulphate – $N : K_1 : S_1$). Taking into account the pepper yield and the accumulation of nitrogen, phosphorus, potassium and sulphur in the fruit, the most favourable fertilisation combination was the combination of an optimal nitrogen dose (170 kg N ha^{-1}) with potassium thiosulphate. The reduction of the nitrogen dose and the treatment of fertilisation with a dose of 128 kg N ha^{-1} with potassium thiosulphate favoured an increase in the vitamin C content of the pepper fruit. The effect of nitrogen dose on tomato fruit yield was modified by the year of the study. Thus, in tomatoes, it is possible to reduce the nitrogen dose depending on weather conditions. At the same time, the addition of potassium thiosulphate is recommended, which has a beneficial effect on the fruit's potassium, phosphorus and sulphur and vitamin C content. There was no significant effect of varying nitrogen and potassium fertilisation on the dry matter content of pepper and tomato fruit, while the effect on calcium, magnesium and extract content was inconclusive.

Keywords: *Capsicum annuum*, *Lycopersicon esculentum*, yield, chemical composition, nutritional value, vegetables

INTRODUCTION

The European Commission proposed the European Green Deal, aiming to reduce plant nutrient losses by at least 50% while preventing soil fertility deterioration and reducing fertiliser use by at least 20% by 2030 [Artyszak and Gozdowski 2020]. Of particular importance for environmental reasons is the reduction

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of nitrogen fertilisation doses. Nitrogen is an essential ingredient for plant growth, so to high restrictions on using nitrogen fertilisers may risk reducing yields.

UAN (urea-ammonium nitrate solution) is increasingly being used to fertilise horticultural and especially agricultural crops. UAN is a highly concentrated nitrogen fertiliser in an aqueous solution of nitrate and urea ammonium nitrate. It is in liquid form, which speeds up the uptake of nitrogen by plants. UAN acts quickly and over time, so plants have a constant nitrogen supply during the growing season. Fertiliser is a solution for ammonium nitrate and urea. In favourable proportions, it contains nitrogen in three forms (ammonium, nitrate and amide). The fertiliser may be applied presowing and top dressing. Applying UAN using a sprayer (coarse droplet tip – droplet diameter over 400 μm) or pouring technique is recommended. Spraying or pouring the fertiliser allows the fertiliser to be evenly distributed. Fine droplet spraying can burn the plants [Bros 2001]. UAN has a high fertiliser efficiency during drought, which has become particularly important in recent years. UAN solution is produced in three N concentrations (28% N, 30% N, 32% N) and is adapted to different transport and storage temperatures. The producer of UAN® fertiliser is Grupa Azoty [https://nawozy.eu/nawozy/azotowe/rsm.html].

Within the framework of a sustainable agriculture system implementing the principles of good agricultural practice (Code of the Best Agriculture Practice) in line with IFA/IFMA declarations, it is recommended, among other things, that highly condensed liquid fertilisers with compositions tailored to the individual nutritional needs of the crops grown be more widely implemented [Górecki 2002]. In line with these trends, UAN has been enriched with sulphur and is offered in the form of two fertilisers: UAN®S – urea ammonium nitrate solution with sulphur, obtained based on urea ammonium nitrate solution and urea solution with ammonium sulphate (contains 26% nitrogen and 3% sulphur); UAN®S 28-5 – obtained based on urea ammonium nitrate solution and ammonium thiosulphate (https://nawozy.eu/nawozy/azotowe-z-siarka/rsms.html).

As part of Project No. POIR.01.02.00-00-0061/17 "Development of a technology for obtaining potassium thiosulphate with the use of blow gases from sulphuric acid production facilities and multicomponent liquid fertilisers based on it", carried out under research topic 43.6 of the "INNOCHEM" sectoral programme, financed from NCBR funds under Measure 1.2 "Sectoral R&D programmes", Priority Axis I "Support for the conduct of R&D works by enterprises" of the Operational Programme Intelligent Development 2014–2020, (Task No. 3) in model studies the optimum N : K (and respectively S) ratio was established for the best efficiency of nitrogen contained in UAN. Experimental research included pot experiments in the first year, which provided the basis for field experiments.

Potassium is among the elements that significantly impact the quality of vegetable and fruit yield [Lester et al. 2010, Mardanluo et al. 2018]. The plant takes up this element rapidly and in large quantities as a monovalent cation supplied to the soil or substrate with potassium fertilisers [Isidora et al. 2008, Pitura et al. 2012]. According to Golcz et al. [2012], "the type of potassium fertiliser used plays an important role in plant nutrition. The supply of potassium to plants in the form of chloride, sulphate or nitrate modifies the chemical composition of plants because the anions accompanying potassium have different functions".

This study evaluated the effectiveness of fertilisation in pepper and tomato cultivation of UAN mixtures with potassium thiosulphate in proportions selected based on a model pot experiment. Tomato and pepper are the primary vegetables of the *Solanaceae* family present in our diet, whose yield and nutritional value are of particular importance to humans.

MATERIAL AND METHODS

The pot experiments were conducted in 2018 provided the basis for field experiments, which started in 2019 and were repeated in the same scheme in 2020. The field experiment was conducted from 2019 to 2020 at the Felin Experimental Farm of the University of Life Sciences in Lublin. The test plants were sweet peppers of the Balta F_1 cultivar (*Capsicum annuum* L.) and tomatoes of the Mirsini cultivar (*Lycopersicon esculentum* Mill.).

The following basic parameters were determined in order to characterise the soils: the granulometric composition – by the laser method [PN-R-04032], the pH value in 1 mol KCl – by the potentiometric method [ISO 10390], total N – by a modified Kjeldahl

method [ISO 11261], the contents of available phosphorus [PN-R-04023] and potassium [PN-R-04022] by the Egner–Riehm method, and sulphur – by the nephelometric method according to the Bardsley and Lancaster's formula [Boratyński et al. 1975]. The soil on which the field experiment was conducted, according to the classification of soils [IUSS 2022] and agronomic categories, is classified into the group: dust, sub–group: silt loam. Granulometric composition: sand fraction – range 2.0–0.05 mm – 21.21% (of which: 2.0–1.0 mm – 0.00%; 1.0–0.5 mm – 0.00%; 0.5–0.25 mm – 0.00%; 0.25–0.10 mm – 1.61%; 0.10–0.05 mm – 19.60%), dust fraction – range 0.05–0.002 mm – 73.32% (including: 0.05–0.02 mm – 42.96%; 0.02–0.002 mm – 30.36%) and silt fraction – range below 0.002 mm – 5.47%.

In 2019, the soil on which the experiment was conducted was characterised by a slightly acid reaction, a total nitrogen content of 0.90 g N kg^{-1} DM and a low abundance in assimilable forms of phosphorus, a medium abundance in assimilable potassium and a very high abundance in assimilable sulphur. In 2020, the soil reaction was also slightly acidic; the content of total nitrogen was, on average, 0.78 g N kg^{-1} DM, the abundance in assimilable forms of phosphorus and potassium was at a medium level, while the abundance in assimilable sulphur was high (Tab. 1). The weather data during the 2019–2020 crop-growing season are shown in Table 2.

The experiment included the following variable factors: nitrogen rate (2 levels: N_1 – optimum nitrogen rate and N_2 – nitrogen rate reduced by 25% from the optimum rate) and fertiliser composition (2 levels: pure UAN – N : K_0 : S_0, UAN with potassium thiosulphate – N : K_1 : S_1).

Phosphorus fertilisation was applied before planting. Fertilisers based on UAN and potassium thiosulphate were applied according to the recommendations established based on the pot experiment performed in 2018. Fertiliser with a ratio of N : K : S, like 1 : 1.1 : 0.7, was applied to cultivate both species. The doses of fertiliser components and their application dates were the same in both years of the experiment (Tabs 3–5).

Before the experiment, the necessary cultivation treatments was carried out and experimental plots of 2.0 m × 1.2 m (area 2.4 m^2) were delineated. The experiment was performed in 3 replicates. Peppers were grown in a plastic tunnel, and tomatoes were planted in the field.

Sweet peppers of the Balta F_1 cultivar were grown from seedlings produced in the vegetation hall of the Institute of Horticultural Production of the University of Life Sciences in Lublin from sowing carried out annually on 18 March. Plants were transplanted into pots on 5–6 April. Seedlings were planted into the ground in a plastic tunnel on 20.05.2019 and 19.05.2020 at a spacing of 40 × 40 cm (10 plants per plot). During the growing season, the plants were fed three times with 0.01% Pionier Mikro Plus (B 0,2%, Cu – 0,1%, Fe 2,0%, Mn 0,8%, Mo 0,05%, Zn 0,3%), and protective treatments were applied in 2019 against pests: spraying with Pirimor WG 500 (pirymicarb) and Mospilan 20 SP (acetamiprid) and against fungal diseases: Topsin M 500 SC (thiophanate-methyl) and Signum 33 WG (boscalid + pyraclostrobin), while in 2020: preparations Mospilan 20 SP and Vertigo 018 EC (abamectin) and against fungal diseases: Switch 62.5 WG (cyprodinil + fludioxonil) and Scorpion 325 SC (azoxystrobin). Drip irrigation was used in pepper and tomato cultivation. Weeds were removed from the plots manually and with the help of rakes.

Pepper fruits were harvested as the fruits matured at the usable maturity stage – in 2019 on six dates: 23.07., 31.07., 4.09., 11.09., 20.09., 9.10., and in 2020 on four dates: 25.08., 2.09., 16.09., 6.10. Immediately after harvesting the peppers, the weight of individu-

Table 1. Soil chemical properties prior to the establishment of the 2019–2020 experiments in the 0–20 cm soil layer

Year	pH	Content			
		g N kg^{-1} DM	mg P kg^{-1} DM	mg K kg^{-1} DM	mg S-SO_4 kg^{-1} DM
2019	5.80	0.90	23.35	148.0	41.06
2020	5.60	0.78	50.51	157.5	38.54

al fruits per plant and the total number of fruits per 1 plant were determined.

Tomato of the Mirsini cultivar was also grown from seedlings, with sowing carried out on 2.04.2019 and 1.04.2020. The seedling was planted in the field on 22.05.2019 and 18.05.2020 at a spacing of 40 × 40 cm (10 plants per plot). During vegetation, plant feeding was carried out three times with Pionier Mikro Plus (0.01%) and protective treatments were also applied: in 2019 – spraying with Mospilan 20 SP against pests, Topsin M 500 SC 0.15% and Signum 33 WG against fungal diseases; in 2020 – preparation Mospilan 20 SP and against fungal diseases: Topsin M 500 SC, Signum 33 WG, Switch 62.5 WG, Scorpion 325 SC, Guaranteed 500 SC.

Tomato fruit was harvested at the usable maturity stage on nine dates in 2019: 27.07., 2.08., 5.08., 9.08., 16.08., 22.08., 29.08., 5.09., 12.09., and in 2020: 13.08., 20.08., 26.08., 3.09., 9.09., 15.09., 24.09., 05.10., 09.10. Immediately after the tomato harvest, the weight of individual fruits per plant and the total number of fruits per 1 plant were determined. Based on the results obtained during pepper and tomato harvest, the yield of plants per unit area was converted.

Plant material samples were taken for chemical analyses at the functional maturity stage. The chemical composition was determined in the plant material obtained, including the content of the essential mineral elements – total nitrogen, phosphorus, potassium, magnesium, calcium and sulphate (VI) sulphur, as well as the content of vitamin C and extract.

Methodology for chemical analyses of plant material: nitrogen and protein content – by Kjeldahl method; phosphorus content – by vanadium-molybdenum method, potassium; magnesium and calcium content – by ASA method after mineralisation of plant material in concentrated sulphuric acid (H_2SO_4) with the addition of perhydrol (H_2O_2); sulphate(VI) content – by nephelometric method after extraction of plant material with 2% CH_3COOH with addition of activated carbon [Grzesiuk 1968]; vitamin C content – by HPLC method [PN-EN 14130:2003]; extract content (%) – using RQ Easy refractometer and Merck test strips.

The results obtained were statistically verified with the ANOVA module (for factorial systems) of the STATISTICA 9.0 PL (StatSoft, Inc.). NIR values were determined using Tukey's HSD test with a significance level of $\alpha = 0.05$ and $\alpha = 0.01$.

RESULTS AND DISCUSSION

The growth and development of pepper and tomato differed in the subsequent years of the study and were modified mainly by weather conditions (Tab. 2). The onset of pepper flowering was similar in both study years (9.06.2019, 10.06.2020), but the onset of fruit set in 2020 was recorded much later than in the previous year (13.06.2019, 30.06.2020). In 2019, the first

Table 2. Mean air temperatures and amount of precipitation in ES Felin during the experiment in 2019–2020 (by Laboratory of Agrometeorology UP Lublin)

Average decade temperature (°C)	Year	Month and decade															
		V			VI			VII			VIII			IX			X
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1
	2019	9.8	13.8	16.2	20.3	23.4	20.7	17.6	18.0	22.3	19.6	20.0	21.1	17.9	13.1	12.6	8.6
	2020	11.7	11.4	12.0	21.4	24.6	23.3	19.6	18.2	19.8	21.9	20.0	19.4				
Mean monthly for 1951–2005			13.0			16.2			17.8			17.1			12.6		7.8
Amount of precipitation (mm)	2019	0.3	72.2	20.0	3.8	5.2	28.1	17.0	2.8	18.1	36.6	61.8	3.9	14.2	4.6	33.1	28.7
	2020	21.0	5.7	77.6	37.0	41.4	89.7	11.9	16.6	0.2	4.6	4.3	36.2				
Mean monthly for 1951–2005			57.7			65.7			83.5			68.6			51.6		40.1

Table 3. Specification of fertilisers used in the field experiment with pepper and tomato

Name of fertiliser	Fertiliser characteristics
UAN	Urea ammonium nitrate solution Liquid fertiliser: N total – 32% (m/m) N-NH_4 – 8% (m/m) N-NO_3 – 8% (m/m) N-NH_2 – 16% (m/m)
SUPER FOS DAR 40	P_2O_5 soluble in mineral acids (total): 41.2% P_2O_5 soluble in neutral ammonium citrate solution: 31.3% P_2O_5 water-soluble: 28.9% Water-soluble CaO 13.1%
Fertiliser based on UAN 32 and potassium thiosulphate:	
N : K : S – 1 : 1.1 : 0.7	Liquid fertiliser: Total N – 9.05% (m/m) Potassium as K_2O – 9.95% (m/m) Sulphur as S – 6.73% (m/m) Sulphur as SO_3 – 16.81 % (m/m) Density – 1,244 g cm^{-3}

Table 4. Application rates and timing of fertilisers for peppers

N : K_0 : S_0		N : K_0 : S_0	
N_1 dose (kg ha^{-1})	170	N_2 dose (kg ha^{-1})	128
P dose (kg ha^{-1})	88	P dose (kg ha^{-1})	88
K dose (kg ha^{-1})	0	K dose (kg ha^{-1})	0
N : K_0 : S_0 ratio	1 : 0 : 0	N:K_0:S_0 ratio	1:0:0
N : K_1 : S_1 fertiliser		N : K_1 : S_1 fertiliser	
N_1 dose (kg ha^{-1})	170	N_2 dose (kg ha^{-1})	128
P dose (kg ha^{-1})	88	P dose (kg ha^{-1})	88
K dose (kg ha^{-1})	155	K dose (kg ha^{-1})	117
N : K_1 : S_1 ratio:	1 : 1.1 : 0.7	N : K_1 : S_1 ratio:	1 : 1.1 : 0.7
Timing of fertiliser applications			
N and K		30% pre-sowing, 30% in 3–4 weeks after planting, twice 20% at 2–3 week intervals	
P		pre-sowing	

harvest of ripe pepper fruit was made on 23.07.2019; in 2020, it was not until 25.08.2020. The start of tomato flowering in 2019 was on 7.06.2019, and the first green fruit was recorded on 17.06.2019. In 2020, tomatoes started flowering slightly earlier (03.06.2020), but the first green fruit appeared later than the previous year (25.06.2020). In 2019, the first harvest of ripe tomato fruit was made on 27.07.2019; in 2020, it was not until 13.08.2020.

A consequence of the differences in the course of plant vegetation due to the prevailing weather conditions was the yield differences recorded between the

Table 5. Application rates and timing of fertilisers for tomatoes

$N : K_0 : S_0$		$N : K_0 : S_0$	
N_1 dose (kg ha^{-1})	150	N_2 dose (kg ha^{-1})	113
P dose (kg ha^{-1})	44	P dose (kg ha^{-1})	44
K dose (kg ha^{-1})	0	K dose (kg ha^{-1})	0
$N : K_0 : S_0$ ratio	1 : 0 : 0	$N : K_0 : S_0$ ratio	1 : 0 : 0
$N : K_1 : S_1$ fertiliser		$N : K_1 : S_1$ fertiliser	
N_1 dose (kg ha^{-1})	150	N_2 dose (kg ha^{-1})	113
P dose (kg ha^{-1})	44	P dose (kg ha^{-1})	44
K dose (kg ha^{-1})	137	K dose (kg ha^{-1})	103
$N : K_1 : S_1$ ratio:	1 : 1.1 : 0.7	$N : K_1 : S_1$ ratio:	1 : 1.1 : 0.7
Timing of fertiliser applications			
N and K		30% pre-sowing, 30% in 3–4 weeks after planting, twice 20% at 2–3 week intervals	
P		pre-sowing	

years of the study for both tomato and pepper, despite growing them in a plastic tunnel.

The pepper fruit yield per plant for the two years averaged 2.03 kg, and in 2020 (1.55 kg), it was significantly lower than in 2019 (2.48 kg) – as in Table 6. In a study by Michałojć and Dzida [2012], an average of 14.3 fruits with a total weight of 1.431 kg were harvested from 1 plant of sweet red pepper Red Knight F_1 grown in a greenhouse, while in other studies, pepper Rebeka F_1 gave a marketable yield of 1.43–1.89 kg [Michałojć and Horodko 2006]. The fruit yield of tomatoes in subsequent years of the study developed differently from that of pepper, even though they belong to the same botanical family (*Solanaceae*). In 2020, tomato yield per plant was almost twice as high (8.15 kg) as in 2019 (4.36 kg) – as in Table 7. The start of tomato vegetation in 2019 was under over-watering conditions, which can be explained by a different response to nitrogen fertilisation than in 2020 (Tab. 2). The manufacturer does not recommend the application of UAN after rain. The peppers were grown in a plastic tunnel, so excess rainfall in May was not detrimental. However, the differentiated fertilisation did not significantly affect the total tomato fruit yield harvested per plant. In 2020, a slightly higher yield, contrary to the previous year, was harvested from plants fertilised with a lower dose of nitrogen (113 kg N ha^{-1}) with potassium thiosulphate (8.83 kg) than with the other fertilisation variants (7.72–8.10 kg on average), but these differences were not statistically confirmed. It is confirmed by Kowalska's [1996] study, which noted the lack of effect of fertiliser nitrogen form (urea, ammonium and nitrate) on tomato fruit yield.

According to Golcz et al. [2012], the success of red pepper cultivation depends on many factors, including mineral fertilisation, especially potassium and nitrogen. In a study conducted in pepper cultivation, the dose of nitrogen did not significantly affect fruit yield when evaluated independently of potassium fertilisation; however, significantly, the highest pepper yield per plant (2.27 kg) was obtained with N fertilisation in the treatment at 170 kg N ha^{-1} with potassium thiosulphate, and the lowest (1.74 kg) with pure UAN fertilisation (without potassium) at a dose of 170 kg N ha^{-1}. This relationship occurred in both years of the study.

Similar relationships were noted in the assessment of yield per unit area**.** The total pepper fruit yield from all crops averaged 8.36 kg m^{-2} for the two years and was significantly lower in 2020 (6.44 kg m^{-2}) than in 2019 (10.28 kg m^{-2}) – as in Table 6. The average marketable fruit yield of red peppers Cyklon, grown in a plastic tunnel from two years of experiments was 2.15 kg m^{-2} [Golcz et al. 2012]. In the present study, differentiated fertilisation did not significantly affect the yield of

Table 6. Influence of nitrogen dose and fertiliser composition on selected pepper fruit yield traits from all harvests in 2019–2020

Specification	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		170	128	$\bar{x}$	170	128	$\bar{x}$	170	128	$\bar{x}$
Pepper fruit yield per plant (kg)	N : K_0 : S_0	2.18	2.62	2.40	1.30	1.57	1.44	1.74	2.10	1.92
	N : K_1 : S_1	2.73	2.39	2.56	1.81	1.51	1.66	2.27	1.95	2.11
	$\bar{x}$	2.46	2.51	2.48	1.55	1.54	1.55	2.01	2.03	2.02
$LSD_{,05}$		A – ns	B – ns	A × B – 0.53	A – ns	B – 0.20	A × B – 0.38	A – ns	B – ns	A × B – 0.365
								A × C – ns	B × C – ns	C – 0.197
The unit weight of pepper fruit (g)	N : K_0 : S_0	228.38	243.22	235.80	208.55	221.09	214.82	218.47	232.16	225.31
	N : K_1 : S_1	249.38	254.20	251.79	214.15	230.62	222.38	231.77	242.41	237.09
	$\bar{x}$	238.88	248.71	243.80	211.35	225.86	218.60	225.12	237.29	231.20
$LSD_{0.05}$		A – ns	B – 13.85	A × B – ns	A – 14.11	B – ns	A × B – ns	A – 9.782	B – 9.782	A × B – ns
								A × C – ns	B × C – ns	C – 9.782
Number of pepper fruit per plant	N : K_0 : S_0	9.63	10.80	10.21	6.30	7.20	6.75	7.97	9.00	8.48
	N : K_1 : S_1	11.00	9.47	10.23	8.63	6.67	7.65	9.82	8.07	8.94
	$\bar{x}$	10.32	10.13	10.22	7.47	6.93	7.20	8.90	8.53	8.71
$LSD_{0.05}$		A – ns	B – ns	A × B – 1.34	A – ns	B – ns	A×B – 1.84	A – ns	B – ns	A × B – 1.471
								A × C – ns	B × C – ns	C – 0.793
Total pepper fruit yield (kg m^{-2})	N : K_0 : S_0	9.11	10.92	10.01	5.42	6.55	5.98	7.27	8.74	8.00
	N : K_1 : S_1	11.39	9.69	10.54	7.54	6.27	6.91	9.47	7.98	8.73
	$\bar{x}$	10.25	10.31	10.28	6.48	6.41	6.44	8.37	8.36	8.36
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 1.880

$\bar{x}$ – mean; ns – no significant differences

peppers. However, in both years of the study, a slightly higher yield was obtained when peppers were grown in the fertilisation treatment of 170 kg N ha^{-1} with potassium thiosulphate (on average 9.47 kg m^{-2}), and the smallest without the addition of potassium thiosulphate (7.27 kg m^{-2}). In a study by Golcz et al. [2012], higher levels of N and K fertilisation significantly increased total red pepper fruit yield by an average of 18%. Nurzyński [1994] reported no significant differences in tomato, pepper, cucumber, lettuce or kale yields when different forms of potassium fertiliser were applied.

The total tomato fruit yield from all harvests in 2020 averaged 33.96 kg m^{-2}, almost double that of 2019 (18.17 kg m^{-2}) – as in Table 7. Significantly higher tomato yield in 2020 was obtained in the trial fertilised with a lower nitrogen rate (113 kg N ha^{-1}) than with 150 kg N ha^{-1} and slightly higher when fertilised with the combination of 113 kg N ha^{-1} with potassium thiosulphate (average 36.77 kg m^{-2}). However, the effect of differential fertilisation on tomato yield was insignificant in the year-independent evaluation. Ddamulira et al. [2019] found that lower

Table 7. Influence of nitrogen dose and fertiliser composition on selected tomato fruit yield traits from all harvests in 2019–2020

Specification	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		150	113	$\bar{x}$	150	113	$\bar{x}$	150	113	$\bar{x}$
Tomato fruit yield per plant (kg)	N : K_0 : S_0	4.37	4.32	4.34	7.96	8.10	8.03	6.17	6.21	6.19
	N : K_1 : S_1	4.44	4.32	4.38	7.72	8.83	8.27	6.08	6.58	6.33
	$\bar{x}$	4.40	4.32	4.36	7.84	8.46	8.15	6.12	6.39	6.26
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 0.513
The unit weight of tomato fruit (g)	N : K_0 : S_0	156.59	151.86	154.23	203.27	203.99	203.63	179.93	177.93	178.93
	N : K_1 :S_1	156.43	153.08	154.76	204.38	213.58	208.98	180.41	183.33	181.87
	$\bar{x}$	156.51	152.47	154.49	203.83	208.79	206.31	180.17	180.63	180.40
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 6.277
Number of tomato fruit per plant	N : K_0 : S_0	28.23	28.10	28.16	39.13	39.30	39.22	33.68	33.70	33.69
	N : K_1 : S_1	28.50	28.37	28.44	38.00	41.03	39.52	33.25	34.70	33.98
	$\bar{x}$	28.37	28.23	28.30	38.57	40.17	39.37	33.47	34.20	33.84
$LSD_{0.05}$		A – ns	B – ns	A×B – ns	A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 2.474
Total tomato fruit yield (kg m^{-2})	N : K_0 : S_0	18.21	17.97	18.09	33.15	33.74	33.45	25.68	25.86	25.77
	N : K_1 : S_1	18.49	18.00	18.24	32.15	36.77	34.46	25.32	27.39	26.35
	$\bar{x}$	18.35	17.98	18.17	32.65	35.26	33.96	25.50	26.62	26.07
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – 2.46	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 1.515

$\bar{x}$ – mean; ns – no significant differences

fertiliser rates negatively affected the growth and yield of cherry tomatoes. They showed that tomatoes responded significantly to nitrogen and potassium fertiliser application by increasing plant height and yield. In a study by Gupta and Sengar [2000], increasing nitrogen and potassium application increased tomato yield and fruit weight. Many authors believe that increasing potassium fertilisation levels can increase tomato fruit yield [Al-Karaki 2000, Yurtseven 2005]. Significant increase in tomato yield with increasing potassium fertiliser rates was reported by Javaria et al. [2012]. According to Ehsan et al. [2010], despite high K levels in the soil, tomato requirements for higher yield could not be met from the native source; hence, the addition of potassium is necessary through fertilisation. Yang et al. [2018] showed that nitrogen fertilisation significantly affected tomato fruit yield, followed by potassium fertilisation and plant density. They found a significant effect of interaction between plant density and fertiliser rate. In a study by Breś and Ruprik [2006], the amount of nitrogen and potassium and the nitrogen/potassium ratio in the nutrient solution significantly affected the yield of small-fruited greenhouse tomato cultivars. Depending on the cultivar, the optimum N : K ratio was in the range of 1 : 1.3–1 : 1.4, while no apparent effect of the applied

nutrient solutions on the nutritional value of the cultivated tomato cultivars was found.

The unit weight of pepper fruit averaged 231.20 g from all harvests from the two years of the study and was significantly higher in 2019 (243.80 g) than in 2020 (218.60 g) – as in Table 6. Fruit with a higher weight (by 12.17 g on average) was obtained using a lower nitrogen rate (128 kg N ha^{-1}). This relationship occurred in both years of the study, but significant differences were recorded only in 2020. Adding thiosulphate to UAN significantly increased the unit fruit weight (by 11.78 g on average). In both years of the study, fruit with the highest weight were harvested from plants fertilised with a lower nitrogen dose (128 kg N ha^{-1}) with potassium thiosulphate, and with the lowest weight from plants fertilised with a higher nitrogen dose (170 kg N ha^{-1}) without potassium fertilisation, although these differences were not statistically significant. In a study by Johnson and Decoteau [1996], increasing the potassium rate in pepper cultivation increased the fruit's biomass, number and weight. El-Bassiony et al. [2010], in the cultivation of sweet pepper 'Kalifornia' showed that increasing the dose of potassium fertilisation to 200 kg had the most significant effect, favourably affecting plant growth, foliage and the highest total yield, as well as fruit size and unit weight. The beneficial effect of potassium fertilisation on pepper fruit yield and quality parameters, including fruit length/diameter ratio, fruit dry matter content, and vitamin C content, was confirmed in a study by Mardanluo et al. [2018].

The varying fertilisation did not significantly affect the unit weight of tomato fruit. The average weight of 1 tomato fruit from all crops and years was 180.40 g and was significantly higher in 2020 (206.31 g) than in 2019 (154.49 g) – as in Table 7. The number of fruits harvested per plant in 2020 averaged 39.37 fruits and was 11.07 higher than in the previous year (28.30 fruits), but there was also no statistically significant effect of varying fertilisation on the total number of tomato fruits harvested per plant. In contrast, in the study by Javaria et al. [2012], the number of tomato fruits per plant increased significantly with increasing potassium fertilisation levels. In a study by Samiullah and Khan [2003], the addition of potassium doubled the number of fruits per 1 plant compared to a crop without potassium fertilisation. Javaria et al. [2012] found that the average tomato fruit weight increased significantly with the addition of potassium compared to no K in fertiliser. Similarly, Padema and Ocala [1999] showed that increased levels of potassium fertilisation led to a significant increase in fruit weight. In their study, unlike tomatoes, pepper responded to varying fertilisation. In both years of the study and the year-independent evaluation, most fruits were harvested from plants grown in the fertilisation combination of 170 kg N ha^{-1} with the addition of potassium thiosulphate (on average 9.82 fruits). On average, 8.71 pieces of fruit were harvested per pepper plant for the two years, with significantly more in 2019 (10.22 fruits) than in 2020 (10.22 fruits) – as in Table 6. The beneficial effect of potassium fertilisation on pepper fruit yield per plant, fruit number and fruit weight was shown by Botella et al. [2017]. Also, in the study by Golcz et al. [2012], higher doses of nitrogen and potassium fertilisation increased the number of pepper fruits. On the other hand, Ortas [2013], investigating the effect of nitrogen and potassium fertilisers, found that pepper and tomato plants responded significantly ($P < 0.01$) to nitrogen fertilisation (100 and 200 kg N ha^{-1}) by increasing plant height, yield and nutrient content, while potassium fertilisers had less effect on these parameters.

The chemical composition of pepper and tomato fruit was assessed at the stage of usable maturity. In pepper fruit, on average, the following were determined: 18.03 g N kg^{-1} DM, 3.36 g P kg^{-1} DM, 30.66 g K kg^{-1} DM, 1.27 g Mg kg^{-1} DM, 2.35 g Ca kg^{-1} DM, 0.72 g S-SO_4 kg^{-1} DM (Tab. 8). The peppers accumulated an average of 7.75% dry matter in the fruit. Depending on the fertilisation combination and the year of the study, this content ranged from 6.56 to 9.14% and was significantly higher in 2019 than in 2020, while differentiated fertilisation did not affect this value. In a study by Kowalska and Sady [2012], the dry matter content of pepper fruit ranged from 6.79 to 10.75%. In a study by Zalewska-Korona et al. [2013], the dry matter content in tomato fruit averaged 5.95% and took values ranging from 5.34% to 6.61%, depending on the cultivar. In the study, they determined an average of 16.48 g N kg^{-1} DM, 5.17 g P kg^{-1} DM, 35.66 g K kg^{-1} DM, 1.54 g Mg kg^{-1} DM, 2.38 g Ca kg^{-1} DM, 0.67 g S-SO_4 kg^{-1} DM (Tab. 9). The dry matter content of tomato fruit in the successive years of the study ranged from 4.98 to 6.55% on average. Different

fertilisation did not significantly affect this trait. More dry matter was determined in tomatoes harvested in 2019 than in 2020. In a study by Kowalska [1996], the form of fertiliser nitrogen had no significant effect on tomato fruit's dry matter, sugars and ascorbic acid content. A beneficial effect of potassium fertilisation on the dry matter content of pepper fruit was reported by Mardanluo et al. [2018].

Nitrogen fertilisation at a higher (optimum) dose in both years of the study significantly increased the total nitrogen content in pepper fruit (on average by 1.24 g N kg^{-1} DM) and tomato fruit (on average by 1.21 g N kg^{-1} DM) compared to the application of a lower nitrogen dose (Tabs 7 and 8). In an evaluation independent of the year of study, adding potassium thiosulphate to UAN increased the N content of pepper fruit (Tab. 8). Significantly, the highest amount of total nitrogen was determined in pepper fruits grown in the combination fertilised with a dose of 170 kg N ha^{-1} with potassium thiosulphate (average 19.65 g N kg^{-1} DM), and the lowest – with a lower nitrogen dose (128 kg N ha^{-1}) with potassium thiosulphate (average 16.85 g N kg^{-1} DM). This relationship occurred in both years of the study. In tomatoes, the response was different (Tab. 9). Irrespective of the nitrogen dose, the addition of potassium thiosulphate decreased the nitrogen content in tomato fruit by 1.13 g N kg^{-1} DM on average. Irrespective of the year of the study, the highest nitrogen was determined in tomato fruit fertilised with pure UAN without the addition of potassium thiosulphate (17.83 g N kg^{-1} DM on average). According to Zawartka et al. [1996], the total N content in tomato fruit did not significantly depend on the amount of potassium used in fertilisation.

Different doses of nitrogen fertilisation did not significantly affect the phosphorus content in pepper fruits, which, depending on the year of the study, ranged from 2.64 to 4.65 g P kg^{-1} DM on average (Tab. 8). On the other hand, in tomato fruits, irrespective of the study year, significantly more phosphorus (on average by 0.72 g P kg^{-1} DM) was determined after fertilisation with the optimal dose of nitrogen (150 kg N ha^{-1}) than with the reduced dose (113 kg N ha^{-1}) – as in Table 8. More phosphorus was determined in pepper fruit (on average by 0.20 g P kg^{-1} DM) and tomato (on average by 0.18 g P kg^{-1} DM) fertilised with UAN with potassium thiosulphate than pure UAN. This relationship occurred in both years of the study, but only in 2020 were the differences statistically significant. The highest amount of phosphorus on average for the two years (5.57 g P kg^{-1} DM) was determined in tomato fruit fertilised with the higher nitrogen dose (150 kg N ha^{-1}) with potassium thiosulphate, and the least (4.66) – with the lower dose of nitrogen (113 kg N ha^{-1}) without thiosulphate. In pepper fruits, the highest amount of phosphorus was also determined after fertilisation with the higher dose of nitrogen (170 kg N ha^{-1}) with potassium thiosulphate (on average 3.88 g P kg^{-1} DM). In 2020, pepper and tomato fruit contained more phosphorus than in 2019.

In 2020, tomatoes accumulated more potassium in fruit, whereas there was significantly less potassium in pepper fruit in 2020 than in 2019. In 2020, more potassium (on average by 1.45 g K kg^{-1} DM) was determined in pepper fruit fertilised with the optimal nitrogen dose (170 kg N ha^{-1}) than with the reduced one (Tab. 8). However, in an evaluation independent of the year of the study, the effect of nitrogen dose on K content in fruit was not significant. The addition of potassium thiosulphate significantly increased the potassium content of the fruit (on average by 1.56 g K kg^{-1} DM) compared to those obtained from a crop fertilised with pure UAN. In a study by Golcz et al. [2008], the varying levels of nitrogen fertilisation had no effect on the nutritional status of pepper plants with this macronutrient, while the potassium content of the leaves was slightly higher at higher fertilisation levels. The increase in potassium content in pepper fruits after potassium fertilisation is confirmed by the study of Shehata et al. [2019]. In our study, the effect of varying fertilisation on potassium accumulation in tomato fruit was similar to that in pepper (Tab. 8). There was no significant effect of nitrogen dose on potassium accumulation in fruit, while more potassium (on average by 1.46 g K kg^{-1} DM) was determined in tomato fruit fertilised with UAN supplemented with potassium thiosulphate. These correlations occurred in both years of the study. In a study [Golcz and Kozik 2004], pepper fruits contained more potassium with increasing potassium levels in the substrate irrespective of the harvest stage.

The calcium content of pepper fruit in subsequent years of the study ranged from 1.09 to 3.95 g Ca kg^{-1} DM on average, with the effect of fertilisation on Ca

Table 8. Effect of nitrogen dose and fertiliser composition on dry matter content (%) and selected components in pepper fruit (g kg^{-1} DM) in 2019–2020

Specification	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		170	128	$\bar{x}$	170	128	$\bar{x}$	170	128	$\bar{x}$
Dry matter content in pepper fruit (%)	N : K_0 : S_0	9.14	8.89	9.02	6.92	6.56	6.74	8.03	7.73	7.88
	N : K_1 : S_1	8.52	8.64	8.58	6.73	6.56	6.64	7.63	7.60	7.61
	$\bar{x}$	8.83	8.77	8.80	6.82	6.56	6.69	7.83	7.67	7.75
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 0.585
Total N content in pepper fruit (g kg^{-1} DM)	N : K_0 : S_0	18.20	19.41	18.81	17.08	16.52	16.80	17.64	17.97	17.81
	N : K_1 : S_1	20.63	17.83	19.23	18.67	15.87	17.27	19.65	16.85	18.25
	$\bar{x}$	19.42	18.62	19.02	17.87	16.19	17.03	18.65	17.41	18.03
$LSD_{0.01}$		A – 0.663	B – ns	A × B – 1.227	A – 0.797	B – ns	A × B – 1.475	A – 0.452	B – 0.452	A × B – 0.803
								A × C – 0.803	B × C – ns	C – 0.452
P content in pepper fruits (g kg^{-1} DM)	N : K_0 : S_0	2.94	2.99	2.97	2.64	4.44	3.54	2.79	3.72	3.26
	N : K_1 : S_1	3.11	3.16	3.14	4.65	2.89	3.77	3.88	3.03	3.46
	$\bar{x}$	3.03	3.08	3.06	3.65	3.66	3.66	3.34	3.37	3.36
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – ns	B – 0.157	A × B – ns	A – ns	B – 0.135	A × B – 0.241
								A×C – ns	B×C – ns	C – 0.135
K content in pepper fruits (g kg^{-1} DM)	N : K_0 : S_0	33.35	34.15	33.75	26.95	25.05	26.00	30.15	29.60	29.88
	N : K_1 : S_1	33.60	34.25	33.93	29.45	28.45	28.95	31.53	31.35	31.44
	$\bar{x}$	33.48	34.20	33.84	28.20	26.75	27.48	30.84	30.48	30.66
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – 1.31	B – 1.31	A × B – ns	A – ns	B – 0.697	A × B – ns
								A × C – 1.240	B × C – 1.240	C – 0.697
Ca content in pepper fruits (g kg^{-1} DM)	N : K_0 : S_0	3.95	3.18	3.57	1.11	1.09	1.10	2.53	2.14	2.34
	N : K_1 : S_1	3.91	2.31	3.11	1.30	1.90	1.60	2.61	2.11	2.36
	$\bar{x}$	3.93	2.75	3.34	1.20	1.50	1.35	2.57	2.13	2.35
$LSD_{0.01}$		A – 0.357	B – 0.357	A×B – 0.662	A – 0.048	B – 0.048	A × B – 0.088	A – 0.157	B – ns	A × B – ns
								A × C – 0.279	B × C – 0.279	C – 0.157
Mg content in pepper fruits (g kg^{-1} DM)	N : K : S	1.61	1.39	1.50	1.04	1.04	1.04	1.33	1.22	1.27
	N : K_1 : S_1	1.48	1.44	1.46	1.11	1.03	1.07	1.30	1.24	1.27
	$\bar{x}$	1.55	1.42	1.48	1.08	1.04	1.06	1.32	1.23	1.27
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – 0.025	B – ns	A × B – 0.047	A – 0.112	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – 0.112
S (VI) content in pepper fruits (g kg^{-1} DM)	N : K_0 : S_0	0.82	0.66	0.74	0.53	0.53	0.53	0.68	0.60	0.64
	N : K_1 : S_1	0.92	0.67	0.80	0.84	0.74	0.79	0.88	0.71	0.80
	$\bar{x}$	0.87	0.66	0.77	0.69	0.64	0.66	0.78	0.65	0.72
$LSD_{0.01}$		A – 0.180	B – ns	A × B – ns	A – 0.027	B – 0.027	A × B – 0.051	A – 0.079	B – ns	A × B – 0.141
								A × C – 0.141	B × C – ns	C – 0.079

$\bar{x}$ – mean; ns – no significant differences

content being ambiguous (Tab. 8). In 2020, inversely to the previous year, more Ca was determined in pepper fruits fertilised with 128 kg N ha^{-1} than with 170 kg N ha^{-1}, while the addition of potassium thiosulphate resulted in a significant increase in calcium content in pepper fruits. In an evaluation independent of the year of the study, more calcium was determined after fertilisation with a dose of 128 kg N ha^{-1} than with 170 kg N ha^{-1}, and the addition of potassium thiosulphate to UAN had no significant effect. In contrast, Mardanluo et al. [2018] reported a reduction in calcium content in peppers with higher levels of K in the nutrient solution. The calcium content of tomato fruit in successive years of the study ranged on average from 1.39 to 3.54 g Ca kg^{-1} DM (Tab. 9). There was no significant effect of the nitrogen dose or the addition of potassium thiosulphate on this trait. Regardless of the year of the study, the most negligible Ca was determined in tomato fruit fertilised with UAN without the addition of potassium thiosulphate.

The magnesium content of pepper fruits took average values of 1.03 to 1.61 g Mg kg^{-1} DM, which was lower in 2020 than in 2019 (Tab. 8). In tomato fruit, Mg content took average values of 1.37 to 1.86 g Mg kg^{-1} DM and was higher in 2020 than in 2019 (Tab. 9). More magnesium was in pepper and tomato fruits fertilised with the optimal nitrogen dose (170 kg N ha^{-1}) than the reduced dose (128 kg N ha^{-1}). The addition of potassium thiosulphate to UAN did not affect the Mg content in pepper fruit, while the highest Mg content in tomato fruit was determined after fertilisation with UAN without potassium thiosulphate (1.65 g Mg kg^{-1} DM). In a study by Jarosz [2006], the type of potassium fertilisation did not significantly affect the chemical composition of tomato fruit.

The effect of differentiated fertilisation on sulphur content in pepper and tomato fruit in both years of the study was analogous (Tabs 8 and 9). Fertilisation with a dose of 170 kg N ha^{-1} increased the sulphate(VI) sulphur content in pepper fruit compared to a dose of 128 kg N ha^{-1}, which did not affect sulphur accumulation in tomato fruit. Adding potassium thiosulphate to UAN increased the sulphur content in the fruit of both studied species (pepper and tomato). In tomato cultivation, this relationship occurred at both nitrogen doses. On average, for two years, tomato fruit fertilised with UAN with potassium thiosulphate contained 0.16 g more sulphate (VI) sulphur than those fertilised with pure UAN (Tab. 9). In pepper fruits, the highest amount of sulphate (VI) sulphur was determined after cultivation in the fertilisation combination of 170 kg N ha^{-1} with potassium thiosulphate (on average 0.88 g $S\text{-}SO_4$ kg^{-1} DM) and the least with pure UAN (0.60 g) – as in Table 8. This relationship occurred in both years of the study. In Kowalska's [2004] study, increasing the sulphate concentration in the nutrient solution increased total sulphur and sulphate content in all tomato parts analysed, regardless of the developmental stage. Breś and Ruprik [2007] found that varying amounts of nitrogen and potassium and the ratios between these elements in the nutrient solution (within the studied range) did not significantly affect the nutritional status of small-fruited tomato cultivars concerning macro and micronutrients.

The content of vitamin C and extract in pepper fruits did not differ significantly between the years of the study. On average, sweet pepper fruits contained 160.37 mg of vitamin C per 100 g of FW (Tab. 10). In a study by Golcz and Kozik [2004], the vitamin C content in sweet pepper was 156–316 mg and varied depending on the nitrogen fertiliser type, potassium fertilisation level and weather conditions. Michałojć and Horodko [2006] determined an average of 131.25 to 141.25 mg of vitamin C per 100 g of FW in the fruits of the pepper Rubin F_1 and showed no apparent effect of differentiated fertilisation on the content of vitamin C and sugars in pepper fruits. In their study, more vitamin C was determined in pepper fruits fertilised with a lower dose of nitrogen (128 kg N ha^{-1}) than with 170 kg N ha^{-1}. This relationship occurred in both years of the study. In contrast, the variation in nitrogen dose did not significantly affect the vitamin C content of tomato fruit in both years of the study, which ranged from 23.13 to 27.47 mg/100 g FW and was higher in 2019 (Tab. 11). In the study by Zalewska-Korona et al. [2013], the average vitamin C content of tomato fruit was 16.02 mg/100 g and ranged from 12.82 to 20.51 mg/100 g, depending on the cultivar. In the study conducted, the addition of potassium thiosulphate to UAN increased the vitamin C content of tomato fruit in both years. On average, for two years, the difference was significant and amounted to

Table 9. Effect of nitrogen dose and fertiliser composition on dry matter content (%) and selected components in tomato fruit (g kg^{-1} DM) in 2019–2020

Specification	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		150	113	$\bar{x}$	150	113	$\bar{x}$	150	113	$\bar{x}$
Dry matter content in tomato fruit (%)	N : K_0 : S_0	6.30	6.55	6.42	5.32	5.47	5.39	5.81	6.01	5.91
	N : K_1 : S_1	6.48	6.50	6.49	4.98	5.39	5.19	5.73	5.95	5.84
	$\bar{x}$	6.39	6.53	6.46	5.15	5.43	5.29	5.77	5.98	5.88
$LSD_{0.05}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – ns A × C – ns	B – ns B × C – ns	A × B – ns C – 0.339
Total N content in tomato fruit (g kg^{-1} DM)	N : K_0 : S_0	16.05	15.68	15.87	19.60	16.80	18.20	17.83	16.24	17.04
	N : K_1 : S_1	16.33	13.81	15.07	16.33	17.17	16.75	16.33	15.49	15.91
	$\bar{x}$	16.19	14.75	15.47	17.97	16.99	17.48	17.08	15.87	16.48
$LSD_{0.01}$		A – 0.541	B – 0.541	A × B – 1.00	A – 0.750	B – 0.750	A × B – 1.387	A – 0.403 A × C – ns	B – 0.403 B × C – 0.716	A × B – 0.716 C – 0.403
P content in tomato fruits (g kg^{-1} DM)	N : K_0 : S_0	5.12	5.26	5.19	5.84	4.06	4.95	5.48	4.66	5.07
	N : K_1 : S_1	4.54	4.70	4.62	6.59	5.17	5.88	5.57	4.94	5.25
	$\bar{x}$	4.83	4.98	4.91	6.21	4.62	5.42	5.52	4.80	5.17
$LSD_{0.01}$		A – ns	B – ns	A × B – 0.377	A – 0.149	B – 0.149	A × B – 0.275	A – 0.110 A×C – 0.195	B – 0.110 B×C – 0.195	A × B – 0.195 C – 0.110
K content in tomato fruits (g kg^{-1} DM)	N : K_0 : S_0	33.33	33.93	33.63	37.03	35.42	36.22	35.18	34.68	34.93
	N : K_1 : S_1	34.57	34.97	34.77	38.35	37.67	38.01	36.46	36.32	36.39
	$\bar{x}$	33.95	34.45	34.20	37.69	36.54	37.12	35.82	35.50	35.66
$LSD_{0.01}$		A – ns	B – 1.130	A × B – ns	A – 1.026	B – 1.026	A × B – ns	A – ns A × C – 1.319	B – 0.742 B × C – ns	A × B – ns 0.742
Ca content in tomato fruits (g kg^{-1} DM)	N : K_0 : S_0	2.83	3.54	3.19	1.55	1.39	1.47	2.19	2.47	2.33
	N : K_1 : S_1	3.54	3.10	3.32	1.49	1.54	1.52	2.52	2.32	2.42
	$\bar{x}$	3.19	3.32	3.26	1.52	1.46	1.49	2.36	2.39	2.38
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – 0.163	A – ns A × C – ns	B – ns B × C – ns	A × B – 0.526 C – 0.296
Mg content in tomato fruits (g kg^{-1} DM)	N : K : S	1.44	1.46	1.45	1.86	1.46	1.66	1.65	1.46	1.56
	N : K_1 : S_1	1.42	1.37	1.40	1.60	1.68	1.64	1.51	1.53	1.52
	$\bar{x}$	1.43	1.42	1.43	1.73	1.57	1.65	1.58	1.50	1.54
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – ns	B – ns	A × B – ns	A – 0.078 A × C – 0.139	B – ns B × C – ns	A × B – 0.139 C – 0.078
S (VI) content in tomato fruits (g kg^{-1} DM)	N : K_0 : S_0	0.67	0.74	0.71	0.48	0.46	0.47	0.58	0.60	0.59
	N : K_1 : S_1	0.86	0.89	0.88	0.62	0.62	0.62	0.74	0.76	0.75
	$\bar{x}$	0.77	0.82	0.79	0.55	0.54	0.55	0.66	0.68	0.67
$LSD_{0.01}$		A – ns	B – 0.067	A × B – ns	A – ns	B – 0.008	A × B – 0.014	A – 0.029 A × C – 0.052	B – 0.029 B × C – 0.052	A × B – 0.052 C – 0.029

$\bar{x}$ – mean; ns – no significant differences

Table 10. Influence of nitrogen dose and fertiliser composition on vitamin C and extract content of pepper fruits in 2019–2020

Specifiction	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		170	128	$\bar{x}$	170	128	$\bar{x}$	170	128	$\bar{x}$
Vit. C (mg 100^{-1} g FW)	N : K_0 : S_0	146.08	161.25	153.67	165.17	164.83	165.00	155.63	163.04	159.34
	N : K_1 : S_1	157.42	166.17	161.80	152.33	169.67	161.00	154.88	167.92	161.40
	$\bar{x}$	151.75	163.71	157.73	158.75	167.25	163.00	155.25	165.48	160.37
$LSD_{0.01}$		A – ns	B – ns	A × B – ns	A – 9.01	B – ns	A × B – 15.92	A – 7.642	B – ns	A × B – ns
								A × C – ns	B × C – ns	C – ns
Extract (%)	N : K_0 : S_0	6.25	6.14	6.20	6.25	6.09	6.17	6.25	6.12	6.19
	N : K_1 : S_1	5.53	6.06	5.80	5.93	5.86	5.89	5.73	5.96	5.85
	$\bar{x}$	5.89	6.10	6.00	6.09	5.97	6.03	5.99	6.04	6.02
$LSD_{0.01}$		A – 0.157	B – 0.157	A × B – 0.269	A – ns	B – 0.122	A × B – ns	A – ns	B – 0.106	A × B – 0.181
								A × C – 0.181	B × C – ns	C – ns

$\bar{x}$ – mean; ns – no significant differences

Table 11. Influence of nitrogen dose and fertiliser composition on vitamin C and extract content of tomato fruits in 2019–2020

Specofocation	Fertiliser content (B)	Year (C)								
		2019			2020			Mean		
		N dose kg ha^{-1} (A)								
		150	113	$\bar{x}$	150	113	$\bar{x}$	150	113	$\bar{x}$
Vit. C (mg 100^{-1} g FW)	N : K_0 : S_0	24.58	26.25	25.42	23.13	24.73	23.93	23.86	25.49	24.68
	N : K_1 : S_1	27.42	27.47	27.45	24.93	24.58	24.76	26.18	26.03	26.11
	$\bar{x}$	26.00	26.86	26.43	24.03	24.66	24.35	25.02	25.76	25.39
$LSD_{0.01}$		A – ns	B – ns	A × B – 1.941	A – ns	B – 0.903	A × B – 1.596	A – ns	B – 1.017	A × B – 1.770
								A × C – ns	B × C – ns	C – 1.017
Extract (%)	N : K_0 : S_0	4.75	5.03	4.89	4.42	4.48	4.45	4.59	4.76	4.67
	N : K_1 : S_1	4.91	4.93	4.92	4.39	4.41	4.40	4.65	4.67	4.66
	$\bar{x}$	4.83	4.98	4.91	4.40	4.45	4.43	4.62	4.72	4.67
$LSD_{0.01}$		A – 0.127	B – ns	A × B – 0.219	A – ns	B – ns	A × B – ns	A – 0.082	B – ns	A × B – 0.141
								A × C – ns	B × C – ns	C – 0.082

$\bar{x}$ – mean; ns – no significant differences

1.43 mg/100 g p.m. Significantly, the least vitamin C was determined in tomato fruit fertilised with pure UAN without thiosulphate (analogously in both years of the study). On the other hand, in the case of pepper, in 2020, the highest amount of vitamin C was determined in the fruit of plants fertilised with the combination of 128 kg N ha^{-1} with potassium thiosulphate (average 169.67 mg–100 g^{-1} św.m.) An analogous trend occurred in 2019. Jarosz [2006] found no significant effect of potassium fertilisation on the content of dry matter, vitamin C and total sugars in tomato fruit. In contrast, El-Bassiony et al. [2010], in the cultivation of California sweet pepper, showed that increasing the potassium fertilisation rate to 200 kg had a beneficial effect on vitamin C accumulation in the fruit.

The extract content in pepper fruit for the two years averaged 6.02% (Tab. 10) and in tomato fruit 4.67% DM (Tab. 11), with more extract in tomato fruit in 2019 than in 2020. The nitrogen dose did not significantly affect the extract content in pepper fruit. However, in 2019, more extract was determined in pepper fruit fertilised with a dose of 128 kg N ha^{-1} than after fertilisation with 170 kg N ha^{-1}, and this trend also occurred in 2020. Also, in tomato cultivation, significantly more extract was contained in the fruit of plants fertilised with a lower nitrogen dose (113 kg N ha^{-1}). Adding potassium thiosulphate to UAN significantly reduced the extract content of pepper fruit (by 0.34% on average). The effect of nitrogen dose on the extract content of pepper fruit was inconclusive. On average, for the two years, the highest extract content was determined in pepper fruits fertilised with pure UAN without adding potassium thiosulphate (6.25%). Adding potassium thiosulphate to UAN did not affect extract accumulation in tomato fruit. In a study by Zalewska-Korona et al. [2013], the extract content of tomatoes averaged 4.38%, taking values from 387 to 4.90%, depending on the cultivar. Breś and Ruprik [2006] found no apparent effect of the applied nutrient solutions of the N : K ratio on the nutritional values of the cultivated tomato varieties. The N : K ratio in nutrient solutions used by different authors is very wide, often depending on the growth stage and development of the tomato, and ranges from 1 : 1.05–1 : 1.1 to 1 : 1.87 [Komosa 2000, Kołota and Biesiada 2002].

CONCLUSIONS

1. Taking into account the pepper yield and the accumulation of nitrogen, phosphorus, potassium and sulphur in the fruit, the most favourable fertilisation combination was the combination of an optimal nitrogen dose (170 kg N ha^{-1}) with potassium thiosulphate. The reduction of the nitrogen dose and the combination of fertilisation with a dose of 128 kg N ha^{-1} with potassium thiosulphate favoured an increase in the vitamin C content of the pepper fruit.

2. The effect of nitrogen dose on tomato fruit yield varied by the year of the study. Thus, in tomatoes, it is possible to reduce the nitrogen dose depending on weather conditions. At the same time, the addition of potassium thiosulphate is recommended, which has a beneficial effect on the fruit's potassium, phosphorus and sulphur and vitamin C content.

3. There was no significant effect of varying nitrogen and potassium fertilisation on the dry matter content of pepper and tomato fruit, while the effect on calcium, magnesium and extract content was inconclusive.

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EFFECTS OF MELATONIN AND TRYPTOPHAN APPLICATIONS ON VIABILITY AND GERMINATION PERFORMANCE OF TOMATO SEEDS DURING AND AFTER ARTIFICIAL AGING

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ABSTRACT

Although the diurnal fluctuations of melatonin (Mel) content in plants and its role in abiotic and biotic stress tolerance are well-documented, little is known about its changes within seeds and its potential effects on seed viability or the aging process. This study aimed to determine how artificial aging, induced by a controlled deterioration test, affects the Mel and tryptophan (Trp) content and seed viability. Furthermore, the study evaluated the effects of Mel and Trp applications on mitigating the impacts of aging in artificially aged seeds. Tomato seeds treated with 250 µM Mel and Trp were artificially aged for up to 8 days through controlled deterioration test after which Mel and Trp changes during ageing and the effect of treatments on seed viability and germination performance was determined. Seeds were also treated with Mel and Trp following artificial ageing in order to determine the effects of Mel and Trp on aged seeds. The positive effects of Mel and Trp applications on seed viability and vigor were particularly evident during and after artificial aging, compared to control seeds. It was observed that in control seeds subjected to controlled deterioration test, Mel and Trp contents exhibited an opposite trend. Applications of Mel and its precursor Trp, before and after artificial aging, significantly slowed down the aging process or alleviated the adverse effects of aging by protecting membrane structures against peroxidation and the accumulation of malondialdehyde (MDA) and H_2O_2. Moreover, indicators of seed deterioration such as electrical conductivity, MDA, and H_2O_2 contents were significantly reduced compared to untreated seeds, while the activities of antioxidant enzymes were boosted. In conclusion, the importance of Mel and Trp applications in preserving seed viability, minimizing storage losses, and slowing seed aging has been demonstrated, suggesting practical applications, particularly in preserving seeds of endangered species or valuable breeding materials.

Keywords: seed aging, controlled deterioration test, seed germination, seed viability, antioxidant enzymes

INTRODUCTION

The acquisition of high-quality seeds and healthy seedlings is a pre-request for successful crop production and cultivation [Wimalasekera 2015, Dwivedi et al. 2021]. The main factors determining seed quality include genetic and physical purity and physiological quality encompassing physical integrity, viability, and vigor. These factors influence the production, development, storage, and transportation of seeds [Hampton 2002, El-Maarouf-Bouteau 2022]. The conditions that seeds experience both before and after harvest are among the main factors affecting seed viability and quality [Dornbos 1995]. Adverse conditions such as relative humidity in storage, temperature, and the presence of oxygen can accelerate the aging process in

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seeds [De Vitis et al. 2020, El-Maarouf-Bouteau 2022]. However, as long as these factors are controlled, long-term storage with minimal loss of viability and vigor is possible [Shelar et al. 2008, Zinsmeister et al. 2020]. The controlled deterioration test has been successfully used by many researchers in recent years to predict field emergence performance and storage longevity, as well as to classify seed lots [Rahman et al. 2019, Zhou et al. 2020, Fatokun et al. 2022]. Today, it has become a recommended vigor test for small-seeded vegetable species by the ISTA Seed Vigor Committee [Powell 2022].

Melatonin (Mel, N-acetyl-5-methoxytryptamine) is an indoleamine whose existence was first identified in the bovine pineal gland by Lerner and colleagues [Lerner et al. 1958]. The discovery of Mel has generated significant interest in the scientific community and has added a new dimension to scientific research. Since its discovery, its presence has been demonstrated in evolutionarily diverse organisms such as unicellular organisms, fungi, algae, bacteria, animals, and plants [Liu et al. 2022]. Additionally, the existence of melatonin has been demonstrated in a wide variety of vegetables, fruits, seeds, grains, medicinal aromatic plants, ornamental, and wild plant species [Madebo et al. 2021, Wu et al. 2021, Altaf et al. 2023a, Muhammad et al. 2024]. In all living organisms, including plants, animals, algae, and bacteria, Mel is synthesized from the amino acid, tryptophan (Trp). Trp serves as the precursor not only for Mel but also for serotonin, a compound found in all plants and animals, and for another plant hormone called indole-3-acetic acid [Khattak et al. 2023, Tiwari et al. 2023]. In plants, Mel acts as a protective antioxidant similar to its role in animals, and it is involved in light/dark signaling. Moreover, Mel plays a significant role in improving tolerance to various environmental stress factors and in the growth and development of plants [Ramasamy et al. 2023, Sharma et al. 2024]. Research on stress tolerance of plants has accelerated with exogenous application of Mel and it has been reported that this molecule improves crop yield by mitigating the negative effects of abiotic stresses on plant growth [Ahmad et al. 2023]. Treatment of plants with Mel have been shown to play a protective role in various species under adverse environmental conditions such as drought [Imran et al. 2021, Altaf et al. 2022], low temperature [Korkmaz et al. 2017a, Korkmaz et al. 2022, Li et al. 2022, Zhang et al. 2023], high temperature [Kuppusamy et al. 2023, Yu et al. 2022], heavy metals [Ali et al. 2023, Altaf et al. 2023b], and salinity [Askari et al. 2023, Guo et al. 2023].

The effective role of Mel in conferring tolerance to various abiotic stress factors in plants has been demonstrated through studies conducted at the seedling or plant tissues. However, limited research exists on the potential effects of Mel on seed storability or seed aging. For instance, studies conducted with pepper, cucumber, and maize seeds have reported that during short-term storage (12 months), Mel content increased in winter months while decreasing in summer months [Kołodziejczyk et al. 2015, Köklü 2016]. On the other hand, Yakupoğlu et al. [2018, 2021] demonstrated that the endogenous Mel and Trp contents of lettuce seeds stored for 24 months changed in a circadian rhythm, and they observed that the Mel and Trp contents changed inversely with each other. That is, during the winter months when Mel content increased, Trp content was detected at its lowest levels. Additionally, similar changes in Mel content were observed in pepper seeds stored for two years, with Mel content increasing during the winter months and decreasing in the summer months. Similarly, in our previous study, we investigated the changes in Mel and its precursor-Trp content in tomato (*Lycopersicon lycopersicum* cv. Rio Grande) seeds stored for an extended period (28 months) under room temperature conditions, which caused significant viability losses [Karaca et al. 2023]. At the end of the research, changes similar to the above-mentioned results were observed in tomato seeds stored for 28 months, highlighting the importance of Mel and Trp applications in preserving viability, minimizing storage losses, and slowing aging in naturally ageing seeds. However, it is not known how the Mel and Trp contents change in artificially aged seeds and to what extent the treatments with these substances affect the deterioration caused by artificial aging. Therefore, in this study, we aim to elucidate the changes in Mel and Trp in artificially aged seeds through controlled deterioration tests and to determine the effects of Mel and Trp applications before and after artificial aging on seed viability. The results obtained from this research are expected to provide us with a better understanding of involvement of Mel and Trp in seed ageing and deterioration.

MATERIALS AND METHODS

Effects of Mel and Trp treatments on tomato seeds before artificial ageing

Plant material and seed treatments. Seeds of Rio Grande tomato (*Lycopersicon lycopersicum*) cultivar were purchased from Istanbul Seed Company, Turkey. Seed moisture content determination was carried out according to ISTA (2005) rules and found to be 8.45%. For Mel and Trp treatments, single layers of tomato seeds (in 100 g batches) were placed in trays between filter papers wetted with 250 µM Mel and Trp solutions and the trays were held at 25 °C in darkness for 24 hours. The concentration of Mel and Trp application (250 µM) was selected based on the results of Karaca et al. [2023]. Dry (untreated) seeds were accepted as control.

Controlled deterioration test. Treated and control seeds were placed between moist paper towels and allowed to imbibe to the weight necessary to reach 24% moisture content. Achievement of the desired weight was determined by periodic weighing. The seeds were then placed in air-tight glass bottles to allow moisture equilibration after which they were subjected to controlled deterioration test. Controlled deterioration test was performed with incubating the bottles at 47 °C in a water bath for varying durations of 0, 2, 3, 4, 6, and 8 days [Basak et al. 2006].

Chemicals and reagents. Mel and Trp along with other chemicals were purchased from Sigma-Aldrich Chemicals. Ten mg Mel and Trp were dissolved in ethanol (1 mL) and distilled water, respectively, then final volume was brought to 10 mL with adding double distilled water to make stock solution. Stock solutions were diluted with the mobile phase [see below] to obtain standard curves when calculating Mel and Trp contents of the seeds. All the measurements and tests were conducted in four replicates.

Measurements and analyzes. To determine the effects of Mel and Trp treatments on tomato seeds aged through controlled deterioration, seed samples were taken at the end of each ageing duration. At the end of each duration, following tests and analyses were conducted.

Extraction and analysis of Mel and Trp were performed according to the method of Korkmaz et al. [2014, 2017a,b] with slight modifications. In brief, 0.25 g of seed and 3 mL of ethyl acetate were shaked in darkness for 17 h at 4 °C in the test tubes. After 20 min of centrifugation at 6,000 *g* and 4 °C the supernatant was transferred to other tubes. The remaining residue was dissolved in methanol (0.5 mL), filtered (0.45 µm), and analyzed with HPLC. To determine Mel and Trp contents HPLC device (Prominance UFLC, Shimadzu) with fluorescence detector and Intersil ODS-2 (250 mm × 4.6 mm) column were used. An excitation and emission wavelength were 280 and 350 nm, respectively. The mobile phase consisted of methanol: 0.1 mM Na_2HPO_4/H_3PO_4 buffer (40 : 60, v/v, pH 4.5) and flow rate was 0.6 mL min^{-1}. The retention times of Trp and Mel were 6.6 and 15.6 min, respectively. Mel and Trp concentrations in each sample were calculated by comparison with the sample peak area with the calibration curves for Mel and Trp. The data obtained were expressed as ng g^{-1} fresh weight (FW).

Tomato seed were germinated in dark in temperature-controlled incubators at 14 ±1 °C (chilling conditions) or 25 ±1 °C (optimum conditions). Four replicates of 50 seeds were placed on filter paper moistened with 5 mL of distilled water in glass petri dishes (9 cm). The appearance of the radicle protrusion (2 mm) was considered sufficient for germination and the number of seeds germinated every day was determined and recorded until the numbers of germinated seeds have stabilized. Final germination percentage (FGP) and mean germination time (MGT) were calculated, from the total number of seed germinated, using Seed Germination v.1.0 software. Because no germination was observed at 14 °C and 25 °C for the seeds that were aged for 4 days and 6 days or more, respectively, FGP and MGT could not be calculated for those treatments.

Seed MDA and H_2O_2 contents were determined according to the method reported in Zhang et al. [2005] and Özden et al [2009], respectively. Additionally, electrical conductivity test was performed according to Vidigal et al. [2011]. For enzyme extractions the method described by Seckin et al. [2010] was followed and total soluble protein contents of the extracts were determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. Catalase (CAT) and peroxidase (POX) activities were determined by the method of Güneş et al. [2007] and Herzog and Fahimi [1973], respectively.

Statistical analysis. The data were subjected to two factor (ageing duration and treatments) analysis of variance (ANOVA) using SAS statistical software program and least significant difference (LSD) test was used to determine the differences between treatments.

Effects of Mel and Trp applications on artificially aged tomato seeds. In the previous study conducted to investigate the effects of Mel and Trp applications on tomato seeds aged through controlled deterioration, the aging duration that reduced the germination percentage of tomato seeds to 60% at low temperature (14 °C), was determined as 3 days. Therefore, in order to examine the effects of Mel and Trp applications on the viability and germination performance of tomato seed, the seeds were subjected to controlled deterioration test for 3 days as explained previously. Following ageing, unaged seeds and seeds aged for 3 days were treated with 0 μM or 250 μM Mel and Trp after which they were dried back to original moisture content. After drying, seeds were germinated in dark in temperature-controlled incubators kept at 14 ±1 °C (chilling conditions) or 25 ±1 °C (optimum conditions). Also, the above tests and analyses were conducted to determine the effects of Mel and Trp applications on seeds after aging.

Statistical analysis. The data were subjected to two factor (treatments and aging) analysis of variance (ANOVA) using SAS statistical software program and least significant difference (LSD) test was used to determine the differences between treatments.

RESULTS

Effects of Mel and Trp applications on tomato seeds before artificial ageing

Seed Mel and Trp contents. The Mel and Trp contents of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications are presented in Table 1. Regarding the effect on seed Mel levels, it was found that one of the two main factors in the study (the treatments) had a statistically significant effect, while the other factor, the aging duration, and the interactions between these factors were found to be non-significant. Upon examining the effect of treatments on seed Mel content, it was observed that Mel application resulted in significantly higher Mel levels compared to both control seeds and seeds treated with Trp. In the seeds treated with Mel, the Mel content was determined to be 5013.72 ng g^{-1} FW, whereas in the control seeds and seeds treated with Trp, it was 2.57 ng g^{-1} FW and 3.03 ng g^{-1} FW, respectively (Tab. 1). Additionally, no significant changes in Mel levels were observed with increasing aging duration; instead, small but insignificant decreases were observed compared to the beginning of aging. There was no significant interaction effect between the two main factors on the seed Mel content. Naturally, seeds treated with Mel exhibited a significantly higher Mel content compared to other treatments; however, statistically, this difference was not found to be significant. At the beginning of the experiment (day 0), the Mel content of seeds treated with Mel was 5295.75 ng g^{-1} FW and it increased slightly as aging progressed but ultimately decreased to 5103.19 ng g^{-1} FW by the end of the study on day 8. Similarly, considering the Mel content of control seeds, it was observed that in seeds aged for 2 days, the Mel content decreased, then increased again, reaching its highest value on day 4, and then decreased again with increasing aging duration (Tab. 1).

When examining the effect of controlled deterioration for different durations on the seed Trp content, it was observed that the Trp levels of Trp-treated seeds were similar to those of control seeds, but lower in seeds treated with Mel. The Trp content was 60.76 ng g^{-1} FW in seeds treated with Trp, 60.64 ng g^{-1} FW in control seeds, and decreased to 45.88 ng g^{-1} FW in seeds treated with Mel. Furthermore, when the Trp levels of the seeds were analyzed depending on different aging periods, it was found that the Trp content increased on the 2nd day of aging, then decreased slightly (on the 3rd and 4th days), but significantly increased with further aging (on the 6th and 8th days) – as in Table 1.

Seed germination. Germination test results conducted at 25 °C revealed that the mean germination time (MGT) of tomato seeds aged through controlled deterioration test for different durations (0, 2, 3, 4, 6 and 8 days) after Mel and Trp treatments increased with the progression of the aging period while FGP decreased (Tab. 2). As no seeds germinated after four days of aging, FGP and MGT could not be calculated for durations exceeding four days; therefore, the re-

Table 1. Mel and Trp contents of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications. Values are means ±standard error (SE, n = 4)

Factors	Mel (ng g^{-1} FW)	Trp (ng g^{-1} FW)
Treatments		
Control	2.57 ±0.4 b	60.64 ±10.46 a
Mel	5013.72 ±92.3 a	45.88 ±8.54 b
Trp	3.03 ±0.3 b	60.76 ±11.71a
LSD $_{(0.05)}$	155.5	11.5
Ageing duration-AD (days)		
0	1767.49 ±752.6	15.47 ±2.04 d
2	1583.7 ±682.6	45.35 ±4.25 bc
3	1621.29 ±690.9	32.82 ±1.45c
4	1651.69 ±710.7	30.06 ±2.15 cd
6	1711.76 ±730.1	57.58 ±4.49 b
8	1702.7 ±727.5	153.29 ±13.54 a
LSD $_{(0.05)}$	–	16.2
Treatments*AD		
Control*0	1.48 ±0.05	10.58 ±0.64
Control*2	0.4 ±0.03	51.56 ±3.82
Control*3	1.9 ±0.31	36.56 ±2.41
Control*4	6.13 ±1.33	31.22 ±4.65
Control*6	3.26 ±0.47	75.58 ±4.77
Control*8	2.28 ±0.59	158.32 ±19.12
Mel*0	5295.79 ±75.9	11.11 ±1.59
Mel*2	4748.43 ±343	32.91 ±4.94
Mel*3	4859.43 ±87.7	27.66 ±0.95
Mel*4	4945.26 ±364.6	23.71 ±1.92
Mel*6	5130.21 ±143.8	54.61 ±3.53
Mel*8	5103.19 ±200.4	125.27 ±2.13
Trp*0	5.21 ±0.36	24.71 ±0.34
Trp*2	2.27 ±0.38	51.57 ±9.06
Trp*3	2.52 ±0.30	34.25 ±1.48
Trp*4	3.68 ±0.21	35.25 ±1.69
Trp*6	1.83 ±0.33	42.54 ±0.8
Trp*8	2.64 ±0.3	176.27 ±27.4
ANOVA		
Treatments	***	*
AD	NS	***
Treatments*AD	NS	NS

NS, *, **, ***, not significant, significant at $P < 0.05$, 0.01 or 0.001, respectively

sults of 6 and 8 days of aging were not included in the statistical analysis. Upon examining the effect of treatments on FGP, it was observed that the germination percentage was higher in seeds treated with Mel and Trp (69.6% and 71.3%, respectively) compared to control seeds (66.1%). Furthermore, when the effect of aging duration on the germination percentage of the seeds was considered, it was found that this effect was significant, with a notable decrease in FGP as the duration increased (Tab. 2). The germination percentage, which was 95.8% on day 0 (prior to controlled deterioration testing), decreased progressively with longer aging periods. It was 86.8% after 2 days of aging, 80.0% after 3 days, and 13.3% by the end of the 4th day. As shown in Table 2, the MGT for control seeds was 7.71 days, while it was 7.23 days for seeds treated with Mel and 7.20 days for those treated with Trp. The effect of different aging durations on MGT was significant, with MGT values increasing as the aging duration extended. Initially (day 0), the MGT was 3.67 days, and it increased to 13.22 days by the end of the 4th day. When examining the interaction of treatments with aging duration, it was observed that seeds treated with Mel and Trp generally had lower MGT values compared to control seeds as the aging duration progressed.

The effects of Mel and Trp applications on the germination performance of seeds aged for different durations using controlled deterioration testing at a low temperature (14 °C) are presented in Table 3. Because no germination was observed after more than 3 days of aging, FGP and MGT could not be calculated for these durations. Therefore, the results for 4, 6, and 8 days of aging were not included in the statistical analysis. FGP for control seeds was 75.2%, while it was significantly higher for seeds treated with Mel and Trp, 81.2% and 84.8%, respectively (Tab. 3). As the aging duration increased, FGP at 14 °C was negatively affected. Before the controlled deterioration (day 0), the germination percentage was 93.5%, which decreased to 82.3% after 2 days of aging and further to 65.3% by the end of the third day. The MGT for control seeds was 14.38 days, while for seeds treated with Mel and Trp, it was lower at 13.84 and 13.87 days, respectively (Tab. 3). Examining the effect of different aging durations on MGT at 14 °C revealed that germination speed decreased as the aging duration increased. Initially (day 0), the MGT was 9.76 days, which increased to the highest value of 16.53 days by the end of the second day and then decreased to 15.79 days by the end of the third day. The interaction of the two main factors in the study on FGP and MGT at 14 °C was also statistically significant. It was observed that FGP decreased with increasing the aging duration across all treatments (control, Mel, and Trp), while MGT increased (Tab. 3).

MDA and H_2O_2 contents and membrane permeability (EC). Mel and Trp significantly impacted the MDA and H_2O_2 contents, as well as the EC values, which indicate membrane integrity, during artificial aging of tomato seeds (Tab. 4). The MDA content of control seeds was 397 nmol g^{-1} FW, whereas the seeds treated with Mel and Trp had significantly lower MDA levels (378 nmol g^{-1} FW and 333 nmol g^{-1} FW, respectively). Initially (day 0), the MDA content of the seeds was 322 nmol g^{-1} FW, and significant increases in MDA content were observed as the aging duration progressed. These results indicated that Mel and Trp treatments significantly reduced the MDA contents of the seeds.

The treatments were found to significantly reduce the H_2O_2 content as well (Tab. 4). The H_2O_2 content in control seeds was 340 nmol g^{-1} FW, while seeds treated with Mel and Trp had lower levels (272 nmol g^{-1} FW and 220 nmol g^{-1} FW, respectively) compared to the control. It was observed that the H_2O_2 content of the seeds significantly increased with prolonged aging, reaching its highest level (346 nmol g^{-1} FW) by the 8th day of aging. When examining the effect of the interaction between the treatments and aging duration on H_2O_2 content, it was found that seeds treated with Mel and Trp generally had lower H_2O_2 content compared to the control seeds as aging progressed, and that H_2O_2 content increased with prolonged aging. At the start of the experiment (day 0), the H_2O_2 content of the control seeds was 273 nmol g^{-1} FW, which rapidly increased after 2, 3, and 4 days of aging, reaching the highest values of 381 nmol g^{-1} FW and 406 nmol g^{-1} FW at the end of 6 and 8 days, respectively. A similar pattern was observed in the H_2O_2 content of seeds treated with Mel and Trp; however, the H_2O_2 content of the treated seeds was lower compared to the control seeds (Tab. 4).

Mel and Trp applications resulted in significant decreases in seed EC values, with the lowest EC value obtained from seeds treated with Trp (Tab. 4).

Table 2. Final germination percentage (FGP) and mean germination time (MGT) at 25 °C of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications. Values are means ±SE (n = 4)

Factors	FGP $_{25}$ (%)	MGT $_{25}$ (days)
Treatments		
Control	66.1 ±8.4 b	7.71 ±0.93 a
Mel	69.6 ±8.6 a	7.23 ±0.96 b
Trp	71.3 ±8.5 a	7.20 ±0.91 b
LSD $_{(0.05)}$	2.93	0.27
Ageing duration-AD (days)		
0	95.8 ±0.8 a	3.67 ±0.12 d
2	86.8 ±1.5 b	7.09 ±0.16 b
3	80.0 ±1.6 c	5.54 ±0.20 c
4	13.3 ±1.1 d	13.22 ±0.17 a
LSD $_{(0.05)}$	3.38	0.32
Treatments*AD		
Control*0	94.0 ±0.8	4.14 ±0.10 g
Control*2	84.5 ±3.4	6.64 ±0.20 de
Control*3	74.5 ±2.4	6.37 ±0.21 e
Control*4	11.5 ±1.7	13.69 ±0.37 a
Mel*0	97.0 ±1.7	3.55 ±0.1 h
Mel*2	86.5 ±1.0	7.12 ±0.32 cd
Mel*3	82.0 ±1.4	5.02 ±0.06 f
Mel*4	13.0 ±1.9	13.22 ±0.15 ab
Trp*0	96.5 ±1.5	3.30 ±0.08 h
Trp*2	89.5 ±2.9	7.50 ±0.10 c
Trp*3	83.5 ±2.1	5.23 ±0.15 f
Trp*4	15.5 ±2.2	12.75 ±0.17 b
ANOVA		
Treatments	**	***
AD	***	***
Treatments*AD	NS	***

NS, *, **, ***, not significant, significant at P < 0.05, 0.01 or 0.001, respectively

Table 3. Final germination percentage (FGP) and mean germination time (MGT) at 14 °C of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications. Values are means ±SE (n = 4)

Factors	FGP $_{14}$ (%)	MGT $_{14}$ (days)
Treatments		
Control	75.2 ±4.8 b	14.38 ±0.83 a
Mel	81.2 ±3.8 a	13.84 ±0.93 b
Trp	84.8 ±2.7 a	13.87 ±1.01 b
LSD $_{(0.05)}$	4.23	0.36
Ageing duration-AD (days)		
0	93.5 ±1.0 a	9.76 ±0.19 c
2	82.3 ±1.7 b	16.53 ±0.15 a
3	65.3 ±2.9 c	15.79 ±0.15 b
LSD $_{[0.05]}$	4.29	0.32
Treatments*AD		
Control*0	92.0 ±1.8 ab	10.53 ±0.11 d
Control*2	78.5 ±3.4 de	16.38 ±0.34 ab
Control*3	55.0 ±1.3 g	16.25 ±0.16 ab
Mel*0	94.5 ±2.1 a	9.57 ±0.11 e
Mel*2	82.5 ±2.2 cd	16.61 ±0.26 a
Mel*3	66.5 ±4.2 f	15.32 ±0.20 c
Trp*0	94.0 ±1.2 a	9.19 ±0.22 e
Trp*2	86.0 ±2.4 bc	16.6 ±0.20 a
Trp*3	74.5 ±2.9 e	15.81 ±0.21 bc
ANOVA		
Treatments	***	**
AD	***	***
Treatments*AD	*	**

NS, *, **, ***, not significant, significant at $P < 0.05$, 0.01 or 0.001, respectively

Additionally, damage to cell membrane integrity increased with prolonged aging, as indicated by the rise in EC values. Initially (day 0), the EC value was 20.01 μS cm^{-1} g^{-1}, which increased with aging and reached the highest value of 48.64 μS cm^{-1} g^{-1} after 8 days of aging. Although the interaction effect between treatment and aging duration on EC values was found to be insignificant, it is evident that seeds treated with Mel and Trp generally had lower EC values compared to control seeds as aging progressed, and EC values increased with prolonged aging.

Catalase (CAT) and peroxidase (POX) enzyme activity. The effects of treatments applied to seeds before controlled deterioration aging on CAT and POX enzyme activities were presented in Table 5. The results indicated that treatments positively influenced the activities of both enzymes. The highest CAT and POX activities were observed in seeds treated with Mel and Trp, whereas the enzyme activities in control seeds were significantly lower. When examining the effects of different aging durations on CAT enzyme activity, there was a significant increase in enzyme

Table 4. MDA, H_2O_2 and EC contents of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications. Values are means ±SE (n = 4)

Factors	MDA (nmol g^{-1} FW)	H_2O_2 (nmol g^{-1} FW)	EC (μS $cm^{-1}g^{-1}$)
Treatments			
Control	397 ±9 a	340 h10 a	39.94 ±2.43 a
Mel	378 ±7 b	272 ±8 b	28.82 ±2.24 b
Trp	333 ±9 c	220 ±16 c	25.95 ±2.1 c
LSD (0.05)	15.0	10.0	2.81
Ageing duration-AD (days)			
0	322 ±16 d	199 ±22 f	20.01 ±2.8 e
2	345 ±9 c	230 ±19 e	24.21 ±2.16 d
3	355 ±8 c	259 ±15 d	26.38 ±1.58 cd
4	376 ±13 b	302 ±12 c	30.03 ±2.22 c
6	399 ±8 a	327 ±12 b	40.15 ±2.64 b
8	417 ±10 a	346 ±14 a	48.64 ±2.05 a
LSD (0.05)	22.0	14.0	3.98
Treatments*AD			
Control*0	351 ±18	273 ±10 fg	30.91 ±5.09
Control*2	367 ±14	304 ±7 de	32.75 ±0.97
Control*3	380 ±8	320 ±13 d	33.25 ±0.96
Control*4	405 ±25	355 ±9 c	34.55 ±5.34
Control*6	424 ±14	381 ±2 b	50.9 ±2.38
Control*8	452 ±9	406 ±8 a	57.29 ±1.04
Mel*0	358 ±11	223 ±6 ij	15.04 ±0.4
Mel*2	353 ±14	233 ±7 hi	20.89 ±2.66
Mel*3	361 ±8	253 ±6 gh	24.14 ±0.85
Mel*4	380 ±21	292 ±4 ef	30.64 ±1.51
Mel*6	401 ±3	312 ±2 de	36.81 ±2.89
Mel*8	414 ±20	319 ±16 d	45.38 ±2.58
Trp*0	258 ±14	101 ±14 l	14.09 ±0.65
Trp*2	315 ±10	153 ±8 k	18.99 ±2.46
Trp*3	324 ±5	203 ±8 j	21.74 ±1.15
Trp*4	344 ±11	260 ±5 g	24.88 ±2.76
Trp*6	372 ±2	289 ±1ef	32.74 ±1.54
Trp*8	386 ±3	311 ±12 de	43.23 ±0.32
ANOVA			
Treatments	***	***	***
AD	***	***	***
Treatments*AD	NS	***	NS

NS, *, **, ***, not significant, significant at P < 0.05, 0.01 or 0.001, respectively

Table 5. CAT and POX enzyme activity of tomato seeds subjected to controlled deterioration for different durations after Mel and Trp applications. Values are means ±SE (n = 4)

Factors	CAT (U mg^{-1} protein)	POX (U mg^{-1} protein)
Treatments		
Control	0.56 ±0.06 b	0.0067 ±0.001 b
Mel	0.71 ±0.07 a	0.009 ±0.001 a
Trp	0.69 ±0.07 a	0.0082 ±0.001 a
LSD (0.05)	0.06	0.0015
Ageing duration-AD (days)		
0	0.77 ±0.07 b	0.0167 ±0.002 a
2	1.21 ±0.04 a	0.0069 ±0.001b c
3	0.73 ±0.04 b	0.007 ±0.001 bc
4	0.56 ±0.04 c	0.0081 ±0.001 b
6	0.38 ±0.02 d	0.0056 ±0 cd
8	0.29 ±0.01 e	0.0036 ±0 d
LSD (0.05)	0.08	0.0021
Treatments*AD		
Control*0	0.5 ±0.05 hi	0.0114 ±0.002 b
Control*2	1.13 ±0.01 bc	0.0073 ±0.002 c
Control*3	0.6 ±0.03 gh	0.0062 ±0 cde
Control*4	0.42 ±0.02 ijk	0.0057 ±0 cde
Control*6	0.39 ±0.02 i-l	0.0058 ±0 cde
Control*8	0.29 ±0.02 kl	0.0035 ±0.001 de
Mel*0	1.01 ±0.06 cd	0.0238 ±0.002 a
Mel*2	1.22 ±0.1 ab	0.0059 ±0.001 cde
Mel*3	0.71 ±0.03 fg	0.0072 ±0 c
Mel*4	0.6 ±0.08 gh	0.0069 ±0.001 cd
Mel*6	0.43 ±0.05 ij	0.0063 ±0 cde
Mel*8	0.32 ±0.02 jkl	0.0041 ±0 cde
Trp*0	0.78 ±0.06 ef	0.0148 ±0.002 b
Trp*2	1.27 ±0.08 a	0.0073 ±0.002 c
Trp*3	0.88 ±0.03 de	0.0076 ±0.002 c
Trp*4	0.65 ±0.05 fg	0.0115 ±0.002 b
Trp*6	0.31 ±0.02 jkl	0.0047 ±0 cde
Trp*8	0.27 ±0.02 l	0.0031 ±0 e
ANOVA		
Treatments	***	**
AD	***	***
Treatments*AD	***	***

NS, *, **, ***, not significant, significant at P < 0.05, 0.01 or 0.001, respectively

activity after 2 days of aging. However, as aging duration increased, substantial decreases in the activity of this enzyme were observed. For POX enzyme activity, a significant decrease was detected with increasing aging duration, likely due to increased stress effects. At the beginning of the experiment (day 0), POX activity was determined to be 0.0167 U mg^{-1} protein, which decreased to 0.0036 U mg^{-1} protein after 6 and 8 days of aging. When the interaction effects of treatments and aging duration on CAT activity of the seeds was examined, it was determined that the highest CAT enzyme activity was measured in seeds treated with Mel and Trp after the 2nd day of aging, while the lowest activity was observed in all treatments aged for 6 and 8 days. Similarly, POX activity was highest at the beginning of aging across nearly all treatments; however, as aging progressed, significant decreases in POX activity were observed.

Effects of Mel and Trp applications on artificially aged tomato seeds

Seed Mel and Trp contents. It has been determined that aging did not have a significant effect on the levels of Mel and Trp in seeds (Tab. 6). However, the treatments applied significantly affected the Mel and Trp contents of the seeds, Mel and Trp levels increasing with Mel and Trp treatments, respectively. There was no significant interaction observed between aging and the treatments on the Mel and Trp levels in seeds.

Seed germination. The statistical analysis revealed significant effects of the main factors (aging factor and treatments) on seed germination at 14 °C (Tab. 7).

Table 6. Mel and Trp contents of tomato seeds after Mel and Trp treatments following artificial aging. Values are means ±SE (n = 4)

Factors	Mel (ng g^{-1} FW)	Trp (ng g^{-1} FW)
Ageing		
– (Non-Aged)	1756.23 ±745.22	24.09 ±3.30
+ (Aged)	1743.17 ±738.40	28.61 ±3.53
LSD $_{(0.05)}$	89.2	7.51
Treatments		
Control	6.09 ±2.04 b	22.60 ±4.43 b
Mel	5226.76 ±59.64 a	20.25 ±2.32 b
Trp	16.24 ±1.41 b	36.21 ±3.35 a
LSD $_{(0.05)}$	109.2	9.20
Ageing*Treatments		
Non-Aged*control	0.88 ±0.26	26.95 ±7.67
Non-Aged*Mel	5250.77 ±53.96	14.95 ±1.77
Non-Aged*Trp	17.03 ±2.61	30.38 ±3.82
Aged*control	11.31 ±1.09	18.25 ±4.49
Aged*Mel	5202.76 ±115.33	25.54 ±1.80
Aged*Trp	15.45 ±1.43	42.04 ±3.88
ANOVA		
Ageing	N.S.	N.S.
Treatments	***	**
Ageing*Treatments	N.S.	N.S.

NS, *, **, ***, not significant, significant at $P < 0.05$, 0.01 or 0.001, respectively

Regarding the effect of aging factor, seeds aged for three days exhibited lower germination percentages compared to seeds without any aging treatment. While the germination percentage of non-aged tomato seeds was 90.8%, germination of seeds aged for three days was 73.0%. Furthermore, the germination percentage in control seeds was 75.3%, whereas seeds treated with Mel and Trp exhibited significantly higher germination rates compared to control seeds (86.5% and 84.0%, respectively). The interaction of two main factors on FGP at 14 °C was also found to be significant, and in all treatments (control, Mel, and Trp), seed germination decreased with the effect of aging. The germination percentage in control seeds without any aging treatment was 88.5%, whereas seeds treated with Mel and Trp showed similar percentages compared to control seeds (93.0% and 91.0%, respectively). However, in control seeds aged for three days, the germination percentage was 62.0%, whereas seeds treated with Mel and Trp had statistically significantly higher percentages compared to control seeds (80.0% and 77.0%, respectively). After the germination test conducted at low temperature (14 °C), the MGT of non-aged seeds was 9.19 days, which increased to 14.97 days when the seeds were aged for three days (Tab. 7). Additionally, the MGT was 12.72 days in control seeds, while in seeds treated with Mel and Trp, it was lower compared to control seeds (11.69 days and 11.83 days, respectively). However, the combined effect of aging and treatments on the MGT of seeds at 14 °C was found to be insignificant.

Table 7. Final germination percentage (FGP) and mean germination time (MGT) of tomato seeds at 14 °C and 25 °C after Mel and Trp treatments following artificial aging. Values are means ±SE (n = 4)

Factors	FGP_{25} (%)	MGT_{25} (days)	FGP_{14} (%)	MGT_{14} (days)
Ageing				
– (Non-Aged)	92.0 ±1.4 a	3.56 ±0.1 b	90.8 ±0.7 a	9.19 ±0.2 b
+ (Aged)	80.6 ±1.4 b	6.50 ±0.2 a	73.0 ±2.8 b	14.97 ±0.2 a
$LSD_{(0.05)}$	3.90	0.22	3.70	0.40
Treatments				
Control	83.3 ±2.5	5.49 ±0.6 a	75.3 ±5.1 b	12.72 ±1.1 a
Mel	88.3 ±2.5	4.64 ±0.5 c	86.5 ±2.8 a	11.69 ±1.1 b
Trp	87.5 ±2.8	4.98 ±0.6 b	84.0 ±3.1 a	11.83 ±1.1 b
$LSD_{(0.05)}$	4.79	0.27	4.53	0.49
Ageing*Treatments				
Non-Aged*control	89.5 ±1.3	3.95 ±0.2 d	88.5 ±0.9 a	9.72 ±0.1
Non-Aged*Mel	92.5 ±3.1	3.36 ±0.1 e	93.0 ±0.6 a	8.91 ±0.3
Non-Aged*Trp	94.0 ±2.6	3.37 ±0.1 e	91.0 ±1.3 a	8.93 ±0.2
Aged*control	77.0 ±1.3	7.02 ±0 a	62.0 ±2.2 c	15.72 ±0.3
Aged*Mel	84.0 ±2.9	5.91 ±0.1 c	80.0 ±2.9 b	14.48 ±0.2
Aged*Trp	81.0 ±1.7	6.58 ±0.2 b	77.0 ±3.4 b	14.73 ±0.2
ANOVA				
Ageing	***	***	***	***
Treatments	N.S.	***	***	**
Ageing*Treatments	N.S.	*	**	N.S.

NS, *, **, ***, not significant, significant at $P < 0.05$, 0.01 or 0.001, respectively

When the germination performance of seeds under optimum conditions (25 °C) was examined, it was observed that only the aging factor significantly affected the germination percentage, whereas the interaction with treatments did not have a significant effect (Tab. 7). The data revealed that seeds aged for three days exhibited a lower germination percentage (80.6%) compared to non-aged seeds (92.0%). Similarly, aging tomato seeds for 3 days significantly affected their germination rate under optimal conditions and resulted in slower germination compared to non-aged seeds. The MGT determined as 3.56 days for non-aged seeds increased significantly to 6.50 days for seeds aged for three days. The applications significantly influenced the MGT of the seeds, with the lowest MGT observed in seeds treated with Mel and the highest in control seeds (Tab. 7). Moreover, the interaction between the two main factors was also found to be significant, indicating that in all treatments (control, Mel, and Trp), the MGT of the seeds increased due to the effect of aging.

MDA, H_2O_2 and EC contents. It has been determined that the aging factor did not have a significant effect on the MDA content of the seeds, and both aged and non-aged seeds exhibited similar MDA contents (Tab. 8). However, the treatments applied significantly affected the MDA content, with Mel and Trp treated seeds having lower MDA contents compared to the control seeds (252 nmol g^{-1} FW and 286 nmol g^{-1} FW, respectively, compared to 390 nmol g^{-1} FW in control

Table 8. MDA H_2O_2 and EC contents of tomato seeds after Mel and Trp treatments following artificial aging. Values are means ±SE (n = 4)

Factors	MDA (nmol g^{-1} FW)	H_2O_2 (nmol g^{-1} FW)	EC (µS $cm^{-1}g^{-1)}$
Ageing			
– (Non-Aged)	293 ±33.9	286 ±48.5 b	30.31 ±4.77 b
+ (Aged)	326 ±20.9	475 ±76.6 a	39.67 ±5.84 a
LSD $_{(0.05)}$	72.0	121.0	6.84
Treatments			
Control	390 ±33.8 a	620 ±85.5 a	57.80 ±3.82 a
Mel	252 ±9.2 b	244 ±44.4 b	22.33 ±3.24 b
Trp	286 ±34 b	278 ±40.3 b	24.84 ±2.57 b
LSD $_{(0.05)}$	88.0	149.0	8.38
Ageing*Treatments			
Non-Aged*control	376 ±70.9	481 ±72.3	51.28 ±4.37
Non-Aged*Mel	230 ±6.9	198 ±39.8	20.54 ±2.63
Non-Aged*Trp	275 ±62.2	180 ±8.6	19.10 ±2.12
Aged*control	405 ±12.9	759 ±126.7	64.31 ±4.53
Aged*Mel	275 ±3.2	291 ±78.6	24.12 ±6.32
Aged*Trp	298 ±37.9	377 ±32.4	30.57 ±2.11
ANOVA			
Ageing	N.S.	***	*
Treatments	*	***	***
Ageing*Treatments	N.S.	N.S.	N.S.

NS, *, **, ***, not significant, significant at P < 0.05, 0.01 or 0.001, respectively

Table 9. CAT and POX enzyme activities of tomato seeds after Mel and Trp treatments following artificial aging. Values are means ±SE (n = 4)

Factors	CAT (U mg^{-1} protein)	POX (U mg^{-1} protein)
Ageing		
– (Non-Aged)	0.48 ±0.03 a	0.0086 ±0.001
+ (Aged)	0.36 ±0.02 b	0.0077 ±0.001
LSD $_{(0.05)}$	0.06	0.02
Treatments		
Control	0.39 ±0.04 b	0.0073 ±0.001
Mel	0.48 ±0.04 a	0.0091 ±0.001
Trp	0.39 ±0.02 b	0.008 ±0.001
LSD $_{(0.05)}$	0.1	0.03
Ageing*Treatments		
Non-Aged*control	0.49 ±0.03 ab	0.0077 ±0.001
Non-Aged*Mel	0.55 ±0.05 a	0.0097 ±0.001
Non-Aged*Trp	0.40 ±0.02 bcd	0.0084 ±0.002
Aged*control	0.30 ±0.03 d	0.0068 ±0.002
Aged*Mel	0.41 ±0.03 bc	0.0086 ±0.002
Aged*Trp	0.39 ±0.04 cd	0.0075 ±0.001
ANOVA		
Ageing	***	N.S.
Treatments	*	N.S.
Ageing*Treatments	*	N.S.

NS, *, **, ***, not significant, significant at $P < 0.05$, 0.01 or 0.001, respectively

seeds). The results presented in Table 8 indicated that the two main factors significantly affected the H_2O_2 content. In non-aged seeds, the measured H_2O_2 content (286 nmol g^{-1} FW) was significantly lower compared to those aged for three days (475 nmol g^{-1} FW). Additionally, it has been determined that the treatments significantly reduced the H_2O_2 content. In the control seeds, the H_2O_2 content was 620 nmol g^{-1} FW, whereas in seeds treated with Mel and Trp, the H_2O_2 content was lower (244 nmol g^{-1} FW and 278 nmol g^{-1} FW, respectively). Regarding the amount of membrane leakage, the EC value significantly increased from 30.31 μS $cm^{-1}g^{-1}$ in non-aged seeds to 39.67 μS $cm^{-1}g^{-1}$ in seeds aged for three days. Additionally, the EC value in control seeds was 57.80 μS $cm^{-1}g^{-1}$, while in Mel and Trp treated seeds, it was significantly lower (22.33 $cm^{-1}g^{-1}$ and 24.84 μS $cm^{-1}g^{-1}$, respectively). Furthermore, the interactions between the main factors (aging and treatments) were found to be insignificant for all three parameters.

Catalase (CAT) and peroxidase (POX) enzyme activity. It has been observed that aging for three days significantly affected the CAT enzyme activity of the seeds, reducing the activity from 0.48 U mg^{-1} protein in non-aged seeds to 0.36 U mg^{-1} protein with aging (Tab. 9). Furthermore, Mel and Trp treatments also significantly affected the CAT enzyme activity and the highest activity of 0.48 U mg^{-1} protein was observed in seeds treated with Mel. In contrast, seeds treated with Trp (0.39 U mg^{-1} protein) and control seeds (0.39 U mg^{-1} protein) exhibited similar enzyme activities. Additionally, significant interaction effects between the main factors affecting CAT activity were observed, except for Trp treatments, where aging

was found to reduce CAT enzyme activity. Regarding another stress enzyme, peroxidase (POX), it was determined that aging and treatments individually or together did not have a significant effect on its activity (Tab. 9).

DISCUSSION

In this study, the effects of changes in Mel and Trp contents in artificially aged tomato seeds through controlled deterioration tests, as well as the effects of Mel and Trp applications before and after aging on seed viability and germination performance, have been elucidated. Seeds treated with Mel and Trp prior to controlled deterioration exhibited higher Mel content compared to control seeds. Additionally, extending the aging period did not result in significant changes in Mel levels; rather, minor, and insignificant fluctuations were observed compared to pre-aging levels.

Mel plays various roles throughout all stages of plant development, including regulating the circadian rhythm [Korkmaz et al. 2017a,b, Yakupoğlu et al. 2021], promoting plant growth and development [Muhammad et al. 2024], and affecting processes from seed aging and germination to seedling growth and fruit ripening [Karaca et al. 2022, Sharma et al. 2024]. The content of Mel in plant tissues is significantly influenced by growing conditions, growth stages, environmental factors, and even the time of day when samples are taken [Arnao and Hernandez-Ruiz 2020, Korkmaz et al. 2022]. Studies on Mel in plants indicate that while this molecule is present continuously, its quantity fluctuates throughout the day, with synthesis rates increasing during darkness [Chang et al. 2021, Gao et al. 2023]. Moreover, the timing of sampling throughout the year is also crucial for determining Mel levels in plant tissues [Yakupoğlu 2016]. For instance, researchers have reported significant seasonal variations in Mel levels in eggplant plant throughout its life cycle [Korkmaz et al. 2017b]. Seasonal changes have also been observed during the 1-year storage of pepper [Korkmaz et al. 2018], corn, and cucumber seeds [Kołodziejczyk et al. 2015]. Additionally, similar results have been found in tomato seeds stored for 28 months [Karaca et al. 2023]. Researchers have suggested that increases in melatonin levels observed during winter months in all four species could indicate an evolutionary acquisition of a defense mechanism to protect the seeds against adverse environmental conditions. Mel is a potent free radical scavenger [Fathi et al. 2023, Muhammad et al. 2022], and high Mel levels serve as an antioxidant source in seeds freshly separated from the parent plant. In short, increased Mel levels under adverse environmental conditions act as a defense mechanism in seeds.

When examining the changes in Trp content, it was observed that seeds treated with Trp had significantly higher tryptophan content, reaching 176.27 ng g^{-1} FW on the 8th day compared to control seeds and other treatments. Additionally, seeds treated with Mel had lower tryptophan content (125.27 ng g^{-1} FW) compared to those treated with Trp on the 8th day. Additionally, an increase in Trp content was observed with aging. The variation in Trp content exhibited generally opposite changes compared to fluctuations in Mel content; that is, when Trp content generally increased in all treatments, Mel content tended to decrease. The results of this study show a similar relationship to the Mel content in tomato seeds stored for 28 months, where Mel levels peaked at the beginning of storage (0 months) and remained at low levels at 12 months (August 2018) and 24 months (August 2019), while Trp content showed increases parallel to the decreases in Mel levels [Karaca et al. 2023]. Similarly, Yakupoğlu et al. [2021] demonstrated that the endogenous Mel and Trp content in lettuce seeds stored for 24 months changed in a circadian rhythm, with Mel content and Trp content varying inversely with each other.

Mel is synthesized from the amino acid Trp through different pathways, with involvement of various enzymes during this synthesis process [Khattak et al. 2023]. Negri et al. [2021] have indicated that enzymes facilitating the transformation of molecules during Mel biosynthesis play a significant role in the changes observed in Mel and Trp levels. In particular, increased activity of certain enzymes along this pathway may enhance the biosynthesis of molecules, but excessive production of some molecules can lead to their accumulation in tissues, thereby negatively affecting the synthesis of other molecules. For instance, the conversion of Trp into serotonin occurs at much higher rates compared to serotonin's conversion into Mel, resulting in generally lower Mel levels observed in tissues. Additionally, the concentration of one mol-

ecule applied may suppress the other [Back 2021], or seeds may not require the biosynthesis of precursor molecules (e.g. lower Trp levels when Mel content is high), all of which could also vary by species or variety.

Treatments with Mel and Trp before controlled deterioration reduced the damage caused by aging and improved germination performance and MGT at both temperatures (14 °C and 25 °C). Moreover, in the continuation of this study, treating the tomato seeds artificially aged under controlled deterioration conditions (3 days at 47 °C with 24% humidity) with Mel and Trp also enhanced seed viability, FGP and MGT. Additionally, Mel and Trp treatments in both studies slowed down seed aging and improved germination performance compared to control seeds. It is well known that controlled deterioration test is a stress test developed to reveal vigor differences among seed lots of small-seeded vegetable species and it is successfully used in predicting field emergence performance, assessing storage life, and classifying the seed lots. For instance, Ermiş et al. [2015] subjected 12 tomato seed lots to controlled deterioration tests at 47 °C and 49 °C with 24% humidity for up to 120 hours and they suggested that early seedling emergence count could be used as an alternative and faster method to assess viability after controlled deterioration. Njie [2015] artificially aged seeds of various vegetable species (tomato, onion, cabbage, lettuce, and carrot) for 2 and 4 months under various temperature and humidity conditions and reported that seed viability decreased by 50% in almost all species after 2 months of storage at 27 °C and 60% relative humidity. It was also noted in the studies that as the aging period increased, the rate of deterioration in seeds also increased. Furthermore, in many studies, priming treatments have been suggested to act as a metabolic repair system that delays aging by organizing cellular structures and cell membranes [Tilden and West 1985].

Although detailed information about the role of Mel and Trp in seed aging is limited, recent studies have documented that Mel and Trp applications enhance germination performance of seeds under stressful conditions. For instance, Korkmaz et al. [2017a] indicated that exogenous Mel applications on pepper improved germination and emergence performance under chilling stress compared to control treatments. In a study investigating the effect of endogenous Mel content on seed germination under chilling stress, it was found that genotypes with high Mel content exhibited significantly better seed germination and seedling emergence under chilling stress conditions [Korkmaz et al. 2022]. García-Cánovas et al. [2024] studied the effects of Mel on germination and seedling growth in in aging sorghum seeds and reported that exogenous application of Mel in aging seeds had a biostimulator effect. Kolupaev et al. [2024] reported that exogenous Mel treatments increased the germination of 4-year-old triticale and rice seeds and this effect was linked to melatonin's regulation of the antioxidant system. Furthermore, it has been reported that Trp applications to pepper seeds under salt stress conditions positively affected germination and seedling emergence performance [Korkmaz et al. 2020]. The results of current research also clearly demonstrate the beneficial effects of Trp and Mel on the germination performance of artificially-aged seeds under stressful conditions.

Lipid structures in cell membranes undergo changes due to oxidation, resulting in alterations in membrane integrity, fluidity, permeability, and structure [Kravić et al. 2021, Li et al. 2022]. Lipids are oxidized by free radicals like H_2O_2, leading to the formation of detrimental products such as MDA. Therefore, determining the levels of H_2O_2 and MDA present in plant tissues and organs provides information about the integrity and functionality of membranes. Additionally, with aging, the permeability (conductivity) of cell membranes that facilitate the exchange of substances increases. Increased membrane permeability means that substances within the seed can leak uncontrollably into the external environment during water uptake, which is an undesired situation. The method used to test this phenomenon, electrical conductivity (EC) testing, is crucial for determining the relationship between aging, viability, and vigor in seeds [Özmen and Kenanoğlu 2024]. Our results have shown that EC, MDA, and H_2O_2 values increased parallel to the aging duration. Furthermore, seeds that did not undergo any aging process had lower EC, MDA, and H_2O_2 values compared to seeds aged for three days. Moreover, seeds treated with Trp and Mel both before and after aging generally exhibited lower EC, MDA, and H_2O_2 levels than control seeds. Similarly, it has been reported that lettuce seeds treated with Mel during a 2-year storage period had lower MDA and H_2O_2 contents compared to control seeds, and during this period, MDA and H_2O_2

levels in seeds were generally lowest when Mel levels were highest [Yakupoğlu et al. 2021]. Köklü [2016] reported that Mel significantly reduced EC, MDA, and H_2O_2 contents in stored seeds compared to untreated seeds. Karaca et al. [2023] also observed that Mel and Trp applications before storage significantly lowered the EC, MDA, and H_2O_2 contents of seeds.

Mel has been reported to directly play a role as an antioxidant in balancing membrane fluidity and lipid peroxidation in biological membranes such as mitochondria, chloroplasts, and plasma membranes, and it is effective in combating stress factors [Chrustek and Olszewska-Słonina 2021, Golding and Lee 2023]. Moreover, Mel regulates and enhances the activities of antioxidant enzymes such as POX, GR (glutathione reductase), SOD (superoxide dismutase), and CAT in plants under stress conditions [Colombage et al. 2023]. In this study, it was observed that pre-treatments before controlled deterioration increased CAT and POX enzyme activities. Additionally, applications of Mel and Trp to tomato seeds artificially aged through controlled deterioration tests resulted in positive improvements in CAT enzyme activity. Furthermore, higher CAT enzyme activity determined in seeds treated with Mel and Trp compared to control seeds is a strong indication that these treatments enhanced the activity of this enzyme. The results of this study are consistent with positive outcomes observed in various species studied, such as lettuce [Yakupoğlu et al. 2021], pepper [Korkmaz et al. 2017a], melon [Castanares and Bouzo 2019], and tomato [Karaca et al. 2023], where Mel applications have been shown to increase the activation of antioxidant enzymes and improve seed germination performance.

CONCLUSION

In this study, the changes in the viability of seeds artificially aged through controlled deterioration following Mel and Trp applications have been demonstrated for the first time. Furthermore, the effects of Mel and Trp applications on seeds aged through controlled deterioration have been identified to alleviate the impacts of aging. The positive effects of Mel and Trp applications on seed viability and vigor have been clearly observed, especially when compared to control seeds. Additionally, due to the stress effects in artificial aging, aging occurred more rapidly due to deformations in the seeds; however, the applications mitigated the severity of damage caused by aging. In controlled deterioration studies, it was found that control seeds without any treatment showed contrasting changes in Mel content compared to Trp content. Specifically, an increase in Mel content in artificially aged control seeds corresponded to a decrease in Trp content. Additionally, applications of Mel and Trp before and after artificial aging have significantly slowed down the aging process or alleviated the negative effects caused by aging by protecting membrane structures against peroxidation, electrolyte leakage, MDA, and H_2O_2 accumulation. Moreover, Mel and Trp applications have been observed to positively enhance the activities of antioxidant enzymes (CAT, POX). In conclusion, it is clear that pre-treatment with Mel and Trp, which serves as a precursor in Mel synthesis, can be a valuable tool to slow down seed aging. This is particularly important for the long-term storage of seeds of endangered species or valuable reproductive materials, highlighting the important practical applications.

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THE EFFECT OF CHITOSAN APPLICATION ON GROWTH, DEVELOPMENT, DECORATIVE VALUE AND YIELD OF *Gladiolus hybridus* Hort. CORMS

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ABSTRACT

Along with roses, carnations, and chrysanthemums, *Gladiolus hybridus* Hort. is an important ornamental plant species cultivated worldwide for cut flowers with long vase life. The study was conducted outdoors, in uncovered soil, in the years 2022–2023. The plant material consisted of daughter corms of five *Gladiolus hybridus* Hort. cultivars: Frizzled Coral Lace, Limoncello, Mon Amour, Nova Lux, and Peter Pears. The study used chitosan with a molecular weight of 6000 g · mol^{-1} at a concentration of 0.4%. The compound was applied by soaking the corms before planting and spraying or watering the plants. The corms were planted into the ground in the third decade of May. During the vegetation period, the course of the development phases was assessed, vegetative and generative traits were measured, and the corm yield was evaluated at the end of cultivation. Chitosan determined the course of the development phases, but this depended on the cultivar traits and the application method. The plants treated with chitosan grew taller and produced more leaves, but they did not differ in the number and diameter of the developed flowers from those not exposed to the biostimulant. Other vegetative and generative traits largely depended on the method of chitosan application. Watering and spraying demonstrated the most beneficial effect of the methods evaluated. Regardless of the method of delivering chitosan to the gladioli, it resulted in a higher weight growth index in the daughter corms.

Keywords: biostimulant, cultivar, cut flower, development phases, morphological traits

INTRODUCTION

Gladiolus hybridus Hort. is one of the most decorative and sought-after ornamental plants, holding a leading position in the floriculture market [El-Naggar and El-Nasharty 2016, Dhakal et al. 2017, Bolagam and Natarajan 2020]. It is called the queen of bulbous plants – the flower of splendour and perfection [Parveen and Katiyar 2020]. It can be grown both in open soil [Kumari et al. 2013, Kumar et al. 2016] and undercover [Singh and Kumar 2017], for cut flowers [Amin et al. 2019, Ashwini et al. 2019] or in flower beds in green areas. Currently, numerous cultivars are available on the market, and they differ significantly in terms of their decorative value [Maurya and Kumar 2014, El-Naggar and El-Nasharty 2016] and the length of the production period. Popularity of this plant is mainly due to its low cultivation requirements [Kumari et al. 2013], high ornamental value [Dhakal et al. 2017, Parveen and Katiyar 2020, Kentelky and Szekely-Varga 2021], and considerable durability after cutting [Maurya and Kumar 2014, Ashwini et al. 2019, Bolagam and Natarajan 2020].

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The growing eco-awareness of ornamental plant producers translates into looking for new solutions to obtain high-yield and good-quality crops while reducing production costs. Such a solution may be using natural substances that stimulate plant growth and development [Kisvarga et al. 2022, Stasińska-Jakubas and Hawrylak-Nowak 2022, Rayanoothala et al. 2024]. In addition to their effect on plants, an important characteristic of these substances is their low level of harmfulness for humans [Malerba and Cerana 2016] or the environment [Li et al. 2020, Korbecka-Glinka and Wiśniewska-Wrona 2021]. The use of chitosan can be just such a solution. Chitosan influences the developmental phases of ornamental plants [Żurawik 2013], stimulates their growth [Atteya et al. 2023, Chen et al. 2023] and also significantly determines the size and the quality of the tuber yield [Żurawik et al. 2017]. This compound can be applied by soaking seeds before sowing [Zohara et al. 2019, Stasińska-Jakubas and Hawrylak-Nowak 2022] or bulbs before planting [Ramos-Garcia et al. 2009, Korbecka-Glinka and Wiśniewska-Wrona 2021]. It may also be used for watering or spraying plants during the growing season [Żurawik et al. 2017]. In addition to the properties of chitosan, such as its molecular weight [Stasińska-Jakubas and Hawrylak-Nowak 2022] and concentration [Byczyńska 2018, Zohara et al. 2019], the authors emphasise the importance of the application method for increasing its effectiveness. Still, they strongly highlight its dependence on the species and cultivar [Malerba and Cerana 2017].

Considering the above, it was deemed advisable to check the effect of chitosan application methods on the course of developmental phases, vegetative and generative traits and progeny tuber yield of selected gladiolus garden cultivars. It was hypothesised that chitosan and its application methods would accelerate flowering, enhance ornamental qualities, and obtain good-quality planting material for gladiolus cultivation without using covers.

MATERIALS AND METHODS

Experimental design

The research was conducted in 2022–2023, in uncovered soil, on experimental plots of the West Pomeranian University of Technology in Szczecin (14°31'E and 53°26'N). The plant material consisted of corms of *Gladiolus hybridus* Hort. reproduced locally in our department and belonging to five cultivars: Frizzled Coral Lace – orange-white, fringed and ruffled (Fig. 1), Limoncello – yellow, ruffled (Fig. 2), Mon Amour – pink-white (Fig. 3), Nova Lux – yellow (Fig. 4), and Peter Pears – salmon, ruffled (Fig. 5). In both years of the study, the corms intended for planting were matched in size (Frizzled Coral Lace 17.7–22.1 g; Limoncello 14.5–17.8 g; Mon Amour 15.7–20.5 g; Nova Lux 27.7–33.8 g; Peter Pears 25.9–27.8 g) and health, without mechanical damage, and had a shape typical of the given cultivar. They were covered with dry, fibrous, and tightly adherent enveloping scales. Before planting, the corms had been stored for five months in a cool room at 5–8 °C and relative humidity of 60–70%. Then, on May 20, 2022, and May 29, 2023, they were planted in beds with a spacing of 25 cm between the rows and 15 cm within the rows, to a depth of 10 cm.

A month before the corms were planted, in both years of the study, 10 unit soil samples from a depth of up to 20 cm were taken from the experimental plot using the Egner's stick. They were then combined into collective samples and used to determine pH in H_2O with the potentiometric method, the CP-315M pH meter (Elmetron, Poland), and salinity with the conductometric method and the CC-411 conductivity meter (Elmetron, Poland). N, P, K, Ca, and Mg content was also assessed. Total nitrogen was determined using the Kjeldahl method after prior mineralisation of the samples in concentrated sulphuric acid with the addition of a selenium mixture. Phosphorus was determined by the colourimetric Egner-Riehm method, potassium and total calcium by flame photometry, and magnesium by atomic absorption spectrometry, using Thermo Scientific iCE 3000 Series AA spectrometer (Thermo Fisher Scientific Inc., USA), after mineralisation of the samples in a 1 : 1 mixture of nitric and chloric acids. The results of the analyses are presented in Table 1. Their values were used to supplement nutrient deficiencies in the soil of the experimental plot to the level recommended for *G. hybridus* Hort. cultivation, that is, 60–120 mg · dm^{-3} $N\text{-}NO_3$, 50–100 mg · dm^{-3} P, 150–250 mg · dm^{-3} K, and 80–110 mg · dm^{-3} Mg [Strojny 1993]. Two weeks before corm planting, the soil was enriched with Azofoska (Inco Group S.A.,

Fig. 1. *Gladiolus hybridus* Hort. Frizzled Coral Lace (photo by P. Żurawik)

Fig. 2. *Gladiolus hybridus* Hort. Limoncello (photo by P. Żurawik)

Fig. 3. *Gladiolus hybridus* Hort. Mon Amour (photo by P. Żurawik)

Fig. 4. *Gladiolus hybridus* Hort. Nova Lux (photo by P. Żurawik)

Fig. 5. *Gladiolus hybridus* Hort. Peter Pears (photo by P. Żurawik)

Poland) compound mineral fertiliser (N 13.6, P_2O_5 6.4, K_2O 19.1, MgO 4.5, B 0.045, Cu 0.180, Fe 0.17, Mn 0.27, Mo 0.040, Zn 0.045) at a dose of 30 g · m^{-2}. The top dressing was performed twice, i.e., before earing and during full flowering, with Azofoska fertiliser at 20 g · m^{-2}.

During the growth and development of plants as necessary, essential care treatments, consisting of systematic weeding, topsoil loosening, and irrigation, were performed. Water deficiency was supplemented by drip irrigation. In both years of the study, there was no need to use chemical plant protection against diseases and pests.

The plants were removed from the soil before the first autumn frosts, in the third decade of October. They were then dried without access to light in a well-ventilated room with a relative humidity of 60–70%, at a temperature of 20–24 °C, for 17 (2022) or 21 days (2023). The dried corms were cleaned of dried shoots, leaves, and roots and wet-treated for 30 minutes in a mixture of 0.5% suspension of Captan 50 WP and 0.6% Topsin M 500 SC. Afterwards, they were dried again for 24 hours in a dark room at 18–20 °C. The corms prepared in this way were placed in storage until the experiments in the following year.

The weather conditions during plant growth and development in 2022 and 2023 were assessed based on data from the Institute of Meteorology and Water Management for the Hydrological and Meteorological Station in Szczecin-Dąbie (Tab. 2).

Table 1. Selected chemical properties of the soil from the experimental plot in the subsequent years of research

Year of study	Nutrient content (mg · dm^{-3})					pH in H_2O	Salt concentration (NaCl g · dm^{-3})
	$N\text{-}NO_3$	P	K	Ca	Mg		
2022	47	38	144	6138	127	7.8	0.30
2023	35	26	152	6156	135	7.5	0.25

Table 2. Distribution of air temperature and precipitation for the Hydrological and Meteorological Station in Szczecin-Dąbie, during the *Gladiolus hybridus* Hort. cultivation in the years 2022–2023, as compared with the multi-year period (1991–2020)

Year of study	Months					
	V	VI	VII	VIII	IX	X
Mean daily temperature (°C)						
2022	14.0	18.6	18.9	20.8	13.1	12.1
2023	12.5	17.6	18.8	18.9	18.0	11.3
Multiyear (1991-2020)	13.6	16.8	18.9	18.5	14.3	9.5
Total rainfall (mm)						
2022	33.0	30.0	68.0	74.0	49.0	22.0
2023	6.6	64.0	67.8	88.0	7.8	56.5
Multiyear (1991-2020)	55.8	60.3	76.2	60.3	47.7	43.5

Chitosan application

The study used chitosan with a molecular weight of 6000 g · mol^{-1} at a concentration of 0.4%. The compound was obtained at the Department of Packaging and Biopolymers of the West Pomeranian University of Technology in Szczecin by controlled deacetylation of free-radical chitin derived from shrimp shells through the continuous addition of hydrogen peroxide with a final concentration of 6.2 mmol to a 2.5% chitosan solution. The depolymerised chitosan had a deacetylation rate of 85% [Bartkowiak 2001]. The chitosan solution was used to soak the corms before planting and later to water or spray the plants. At the beginning of the experiment, the corms were soaked in chitosan or tap water and treated for 30 minutes the day before the planting date. After this treatment, the corms were dried for 24 hours in a dark room at 22 °C and a relative humidity of 50%. In the experiments where the plants received chitosan by watering, a biostimulant solution was applied directly to loosen and moist soil near the plants. In other treatments, the chitosan solution was applied using a handheld pressure sprayer to mist both sides of the leaf blade. The initial application took place at the two-leaf stage, with subsequent treatments repeated every 7 days, applying 10 ml of the solution per plant. In total, chitosan was applied 20 times during the growing season. Regardless of the application method, it was dissolved in tap water on the day of application. The control group consisted of plants that were not treated with chitosan.

Plant observations and measurements

The number of days of cultivation was determined as follows: from planting the corms to the start of emergence, from the beginning of emergence to the start of earing, and from the start of earing to the end of flowering. The observations were carried out every 2 days.

The measurements of morphological traits were made during full flowering, and the following parameters were determined: the height of the plants from the ground level to the highest point, the number of developed shoots and leaves on the main shoot, and the plant in general. The leaf greenness index was determined using the Chlorophyll Meter SPAD-502 (Minolta, Japan), which reads SPAD (soil plant analysis development) units. This index is closely correlated with chlorophyll content, as described by Zawadzińska and Salachna [2024]. The SPAD meter is a reliable tool for assessing plant nutritional status and identifying nitrogen deficiencies during later stages of growth [Żurawik 2020]. The measurements were made on 6 mm^2 in the central region of the first properly developed leaf.

The measurements of generative traits were made when the first flowers developed in the inflorescence of successive flowering plants. The following parameters were measured: the length of the inflorescence shoot from its base to its highest point, the inflorescence length, the number of flowers developed, and the diameter of the first flower in the inflorescence. The number of developed inflorescence shoots was also determined at the end of the flowering period.

After drying and cleaning the corms, their yield was evaluated by calculating the number and weight growth indices.

Statistical analysis

The experiments included 20 factors, i.e., 5 cultivars × 4 chitosan application methods. All variants included three repetitions, 8 corms each. A total of 480 plants were evaluated in the study.

The results reflecting chitosan's effects on the developmental phases were processed based on mean values. All statistical analyses were performed using the Statistica Professional 13.3 package (TIBCO StatSoft, Palo Alto, CA, USA). The data on vegetative and generative traits and corm yield were subjected to a two-way analysis of variance (ANOVA) in a complete randomisation system in subsequent years of the study and as a two-year synthesis. The means were compared with Tukey's test for a significance level of $\alpha < 0.05$.

RESULTS AND DISCUSSION

The course of the development phases

The available literature lacks information on the effect of chitosan on the growth and development of ornamental geophytes. Unfortunately, many authors who use this biopolymer do not determine its basic physical and chemical properties, such as molecular weight, which makes it difficult, if not impossible, to compare the obtained research results. The effect of chitosan on the course of the emergence phase depends on the conditions prevailing during plant cul-

tivation [Żurawik 2013]. According to this author, chitosan with a molecular weight of 10 000 g · mol^{-1} and a 0.2% concentration, applied before planting the bulbs of *Freesia* × *hybrida* Lisa, Bon, and Silver Beach, growing in an air-conditioned chamber, with constant temperature and humidity of the substrate, did not determine the date of the bulb germination. On the other hand, changing light, temperature, and humidity slightly delayed the emergence of the main and secondary shoots in Silver Beach freesia. Ramos-Garcia et al. [2009] stated that soaking the corms of *Gladiolus* sp. Blanca Borrego in a 1.5% solution of Biorent before planting accelerated their germination. In our study, soaking the corms before planting in a 0.4% chitosan solution with a molecular weight of 6000 g · mol^{-1} accelerated the emergence phase, but this varied significantly for different cultivars (Fig. 6). In the present experiment, gladiolus cultivars differed during this development phase, which was also observed in other studies [Thakur et al. 2015]. Regardless of the use of chitosan, the corms of Nova Lux were the first to germinate (12.4 days), followed by those of Mon Amour (21.2 days), Fizzled Coral Lace (22.0 days), Peter Pears (22.9 days), and Limoncello (23.4 days). Gladiolus sprouting after 5.6 to 19 days, and the longer emergence period is due to a delayed planting date [Thakur et al. 2015]. In the case of cultivars with a longer sprouting period, i.e., more than 20 days, chitosan accelerated sprouting from an average of 2.2 days in Peter Pears to 5 days in Limoncello. No such dependencies were found for the Nova Lux (Fig. 6).

Soaking corms of *Freesia* × *hybrida* Gompey in a chitosan solution accelerated the formation of inflorescences [Salachna and Zawadzińska 2014], while according to Żurawik et al. [2017], earlier flowering also depended on the frequency and method of this compound application. In the present study, treating gladioli with chitosan shortened their vegetative phase by accelerating the earing (Fig. 6). Regardless of the cultivar, the gladioli treated with chitosan began to ear on average 4.5 days earlier than the control plants. According to Żurawik [2013], the positive effect of chitosan on flowering depends on the length of the cultivation period. Our studies did not confirm this dependency. Earlier earing of the gladioli plants was visible in the cultivars during the shortest and the most extended production period. In the present experiment, the effect of chitosan largely depended on cultivar traits. In the case of earing, it was the weakest for Fizzled Coral Lace and Nova Lux (3.1 days) and the strongest for Limoncello (10.6 days). The Chitosan application method also significantly affected the time of earing. The watered or sprayed plants of Fizzled Coral Lace, Limoncello, Mon Amour, and Peter Pears eared earlier than those obtained from the soaked corms. A different response to the compound application methods was observed in Nova Lux, which may be due to a significant influence of the cultivar traits on the course of this development phase [Thakur et al. 2015].

According to Żurawik et al. [2017], chitosan with a molecular weight of 8000 g · mol^{-1}, prolonged flowering of *Freesia* × *hybrida* Summer Beach regardless of its application method. In the present study, cultivars Fizzled Coral Lace, Limoncello, Mon Amour, and Peter Pears treated with chitosan in any manner flowered longer compared to untreated plants. However, this effect was not observed in Nova Lux (Fig. 6).

Vegetative traits

Treating plants with chitosan positively affected their growth [Van et al. 2013, Malerba and Cerana 2017, Fahmy and Nosir 2021]. The compound increased plant height, but its impact depended on the method of its application (Tab. 3). Spraying *Eleusine coracana* [Sathiyabama and Manikandan 2021], *Calendula officinalis* [Akhtar et al. 2022] or *Withania somnifera* [Jacob et al. 2023] plants stimulated their growth. In the present experiment, the plants sprayed or watered with chitosan were taller than the control group. The differences were insignificant and ranged from 5.2% to 5.4%. The increase in the height of plants watered with chitosan may be due to the effect of chitosan nanoparticles on their biophysical characteristics, among others, the ability to absorb nutrients from the soil [Van et al. 2013]. The height of gladiolus plants also depends on their cultivar traits [Kumar et al. 2016]. The cultivars compared in this experiment also differed in terms of this trait, with Nova Lux being the tallest and Frizzled Coral Lace the shortest. Nova Lux gladioli sprayed with chitosan during the growing season were the tallest, and Frizzled Coral Lace plants grown from the corms soaked in the biopolymer before planting were the shortest (Tab. 3).

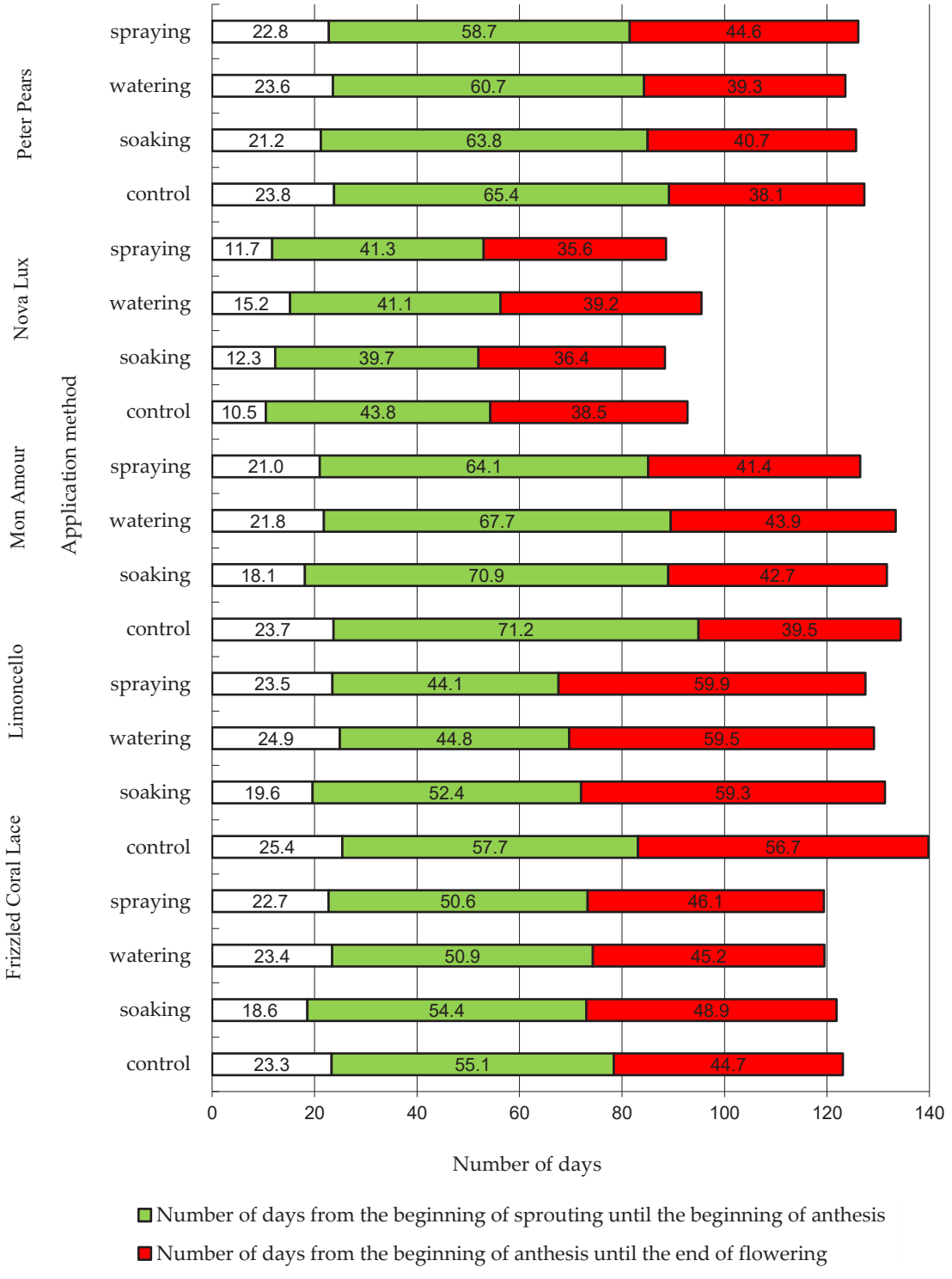

Fig. 6. The number of days from planting the corms to the end of flowering, depending on the *Gladiolus hybridus* Hort. cultivar and chitosan application method

Table 3. Vegetative traits of *Gladiolus hybridus* Hort. depending on the cultivar and chitosan application method (mean for the years 2022–2023)

Trait	Cultivar	Application method				Mean
		I	II	III	IV	
Plant height (cm)	Frizzled Coral Lace	81.0fg	80.0g	82.9efg	82.9efg	81.7C
	Limoncello	84.9defg	87.9defg	89.4def	91.5de	88.4B
	Mon Amour	87.7defg	91.9de	90.1def	93.2cd	90.7B
	Nova Lux	102.3bc	105.1ab	111.9a	107.0ab	106.6A
	Peter Pears	87.5defg	89.5def	93.1cd	91.8de	90.5B
	Mean	88.7B	90.9AB	93.5A	93.3A	91.6
Number of shoots (pcs.)	Frizzled Coral Lace	1.08de	1.00e	1.25cde	1.38cde	1.18C
	Limoncello	1.88abcde	2.42a	2.13abc	2.37ab	2.20A
	Mon Amour	1.17de	1.46bcde	1.37cde	1.35cde	1.34C
	Nova Lux	1.46bcde	1.63abcde	1.96abcd	1.88abcde	1.73B
	Peter Pears	1.25cde	1.46bcde	1.51abcde	1.84abcde	1.52BC
	Mean	1.37B	1.59AB	1.64AB	1.76A	1.59
Number of leaves set on main shoot (pcs.)	Frizzled Coral Lace	8.73ab	8.78ab	8.71ab	8.86ab	8.77AB
	Limoncello	7.63ab	8.72ab	8.18ab	8.70ab	8.31BC
	Mon Amour	7.36b	8.19ab	8.03ab	8.44ab	8.01C
	Nova Lux	7.29b	8.76ab	8.18ab	8.68ab	8.23BC
	Peter Pears	8.87ab	9.10a	8.97a	9.17a	9.03A
	Mean	7.98B	8.71A	8.41AB	8.77A	8.47
Total number of leaves per plant (pcs.)	Frizzled Coral Lace	9.20d	8.78d	10.42cd	11.77cd	10.04C
	Limoncello	13.93abcd	21.04a	17.03abc	20.02ab	18.01A
	Mon Amour	8.55d	11.54cd	10.65cd	11.29cd	10.51C
	Nova Lux	10.60cd	13.94abcd	15.77abcd	15.68abcd	14.00B
	Peter Pears	11.05cd	13.07bcd	13.31bcd	16.84abc	13.57B
	Mean	10.67B	13.67A	13.44A	15.12A	13.22
Greenness index of leaves (SPAD)	Frizzled Coral Lace	62.8cd	62.8cd	65.2abcd	64.7abcd	63.9B
	Limoncello	62.2d	62.5cd	64.1bcd	64.1bcd	63.2B
	Mon Amour	62.7cd	62.4cd	65.4abcd	64.8abcd	63.8B
	Nova Lux	64.1bcd	62.7cd	65.3abcd	64.2bcd	64.1B
	Peter Pears	69.0abc	69.8ab	70.9a	71.2a	70.2A
	Mean	64.2AB	64.0B	66.2A	65.8AB	65.0

I – control, II – soaking, III – watering, IV – spraying
A, B – for the main factor, a, b – for the interaction
Means marked with the same letters do not differ significantly at $\alpha = 0.05$

Regardless of the cultivar, chitosan application by spaying resulted in an increased number of shoots compared to untreated plants, with a maximum difference of 28.5%. Among the analysed cultivars, Limoncello exhibited the highest shoot production, whereas Frizzled Coral Lace and Mon Amour had the lowest. The study showed cultivar-specific responses to chitosan application methods. Specifically, plants of Limoncello demonstrated the most remarkable shoot proliferation when grown from corms soaked before planting, whereas Frizzled Coral Lace plants subjected to the same treatment exhibited the lowest shoot proliferation (Tab. 3).

Literature reports are inconclusive regarding the effect of chitosan on the number of leaves produced by various species of ornamental plants. Spraying *Pelargonium* × *hortorum* twice with 1 mg · dm^{-3} of chitosan does not determine the number of leaves the plants develop [Liu 2023]. However, a positive effect of chitosan on the number of leaves was demonstrated in the rose Anglina [Ali and Asal 2023]. In *Eucomis bicolor*, this effect depended on chitosan concentration [Byczyńska 2018]. In our experiment, the gladioli obtained from the corms soaked in chitosan and those sprayed with this compound developed 9.1% and 9.9% more leaves on the main shoot, respectively, but only if compared to the control plants (Tab. 3). The Peter Pears plants had the most considerable number of leaves, while those of Mon Amour were the fewest. A significant dependency was demonstrated between the assessed cultivars and the chitosan application methods. Peter Pears plants grown from the corms soaked in chitosan, watered, or sprayed with it developed more leaves only as compared to the control Nova Lux gladioli.

Chitosan also significantly determined the total number of leaves developed by the gladioli. Regardless of the cultivar, the plants treated with chitosan produced 26% to 41.7% more leaves than the control ones. Limoncello plants developed the largest, Frizzled Coral Lace, and Mon Amour had the smallest total number of leaves. The most significant number of leaves was observed in Limoncello plants obtained from the corms soaked in chitosan before planting, and the lowest in the control Mon Amour gladioli. This significant difference amounted to 146.1% (Tab. 3).

Spraying *Rosa bourborniana* Gruss-an-Teplitz with chitosan [Arshad et al. 2022] and treating *E. bicolor* bulbs with this compound before planting [Byczyńska 2018] intensified the green colour of their leaves. Our study did not confirm this, as the SPAD index depended mainly on the cultivar. Compared with all other evaluated cultivars, the leaves of Peter Pears plants had the highest greenness index. Regardless of the cultivar, the SPAD index was higher in the plants watered with chitosan than in those grown from the corms soaked before planting. The cultivars evaluated in the study responded differently to the chitosan application method. The most intensely green leaves were noted in the Peter Pears plants sprayed or watered with chitosan, and the lowest SPAD index was observed in the control Limoncello plants (Tab. 3).

Generative traits

According to Byczyńska [2018], soaking *E. bicolor* bulbs before planting induced an increase in inflorescence length. In our study, longer inflorescences were observed in the plants sprayed with chitosan and control ones than those watered with the compound or grown from the corms soaked in it (Tab. 4). The inflorescence length of gladiolus can vary depending on the cultivar, as reported by Thakur et al. [2015]. The Mon Amour plants had the longest inflorescences, while in Nova Lux they were the shortest.

Chitosan spraying significantly affected the number of inflorescences obtained in *Matricaria recutita* [Abdul-Hafeez and Ibrahim 2021] and the number of flowers in *R. bourborniana* Gruss-an-Teplitz [Arshad et al. 2022]. Additionally, a dependence was found between the diameter of *Polianthes tuberosa* flowers and chitosan concentration [Alsanam and Salih 2021]. In the present study, the treatment of gladioli with 0.4% chitosan with a molecular weight of 6000 g · mol^{-1} did not significantly affect the number of developed flowers or their diameter (Tab. 4). These traits depended mainly on the cultivar, which other studies also confirmed [Thakur et al. 2015]. Reports on the effects of soaking plant bulbs in chitosan on flower production remain inconclusive. Ramos-Garcia et al. [2009] observed a positive impact on flower number in gladioli Blanca Borrego following treatment with a 1.5% Biorent preparation. However, Byczyńska [2018] did not confirm this effect in *E. bicolor*. In the present study, Limoncello plants produced the highest number of flowers per inflorescence, whereas Frizzled Coral

Lace exhibited the lowest. Conversely, flowers with larger diameters were recorded in Frizzled Coral Lace, Nova Lux, Peter Pears, and Mon Amour, while Limoncello plants produced flowers with smaller diameters.

Corm yield

Treating *E. bicolor* bulbs before planting with chitosan at 50 and 100 mg · dm^{-3} increased their weight [Byczyńska 2018]. Chitosan of a molecular weight of 7000 or 10 000 g · mol^{-1} used during the rooting of *Eucomis comosa* Sparkling Burgundy leaf seedlings contributed to increasing the weight of adventitious bulbs [Kukla and Żurawik 2022]. Our study also showed that 0.4% chitosan with a molecular weight of 6000 g · mol^{-1} increased the corm weight growth index, regardless of the application method (Tab. 5). Concerning the plants not treated with this compound, the significant differences ranged from 21% to 41.9%. Żurawik and Bartkowiak [2009] linked the size of this index in garden freesia with the chitosan application method. In the present study, soaking the bulbs before planting had a stronger effect on this trait, only concerning spraying the plants during the growing season. Similar relationships were demonstrated by Kukla and Żurawik [2022] for rooting of *E. comosa* Sparkling Burgundy leaf seedlings. Regardless of the chitosan application method in the present experiment, the highest corm weight growth index was observed in the Nova Lux plants and the lowest in the Peter Pears ones. The investigated cultivars responded differently to the chitosan application methods. The highest corm weight growth index was found in the Mon Amour and Nova Lux plants obtained from the corms soaked before planting, whereas the lowest was in the sprayed Peter Pears plants. These significant differences amounted to 155.8% (Tab. 5).

Byczyńska [2018] observed that the effect of chitosan on the number of developed bulbs in *E. bicolor* depended on its concentration. Moreover, Biorent at a concentration of 1.5% increased the number of daughter corms developed by *G. hybridus* Hort. [Ramos-Garcia et al. 2009]. According to Żurawik [2013], chitosan with a molecular weight of 10 000 g · mol^{-1} stimulated the growth of a more considerable number of buds in garden freesia Silver Beach, which resulted in a rise in the bulb count rate by up to 44.3%. No differences of this magnitude were found in the present study using 0.4% chitosan with a molecular weight of 6000 g · mol^{-1}. Compared with the control plants, a 28.5% higher corm count increase rate was obtained only from spraying the plants with this compound. The rate was the highest in Limoncello plants and the lowest in Mon Amour and Frizzled Coral Lace. The differences were 64.2% and 86.4%, respectively (Tab. 5). There were also differences in the response of the cultivars to the chitosan application methods. The highest corm count increase rate was observed for the Limoncello plants when the corms were soaked before planting, and the lowest for the Frizzled Coral Lace plants for the same chitosan application method. The difference was significant and amounted to 142% (Tab. 5). The increase in the corm weight growth index was due to an increase in the assimilation area, which resulted from the gladioli developing a more significant number of leaves. On the other hand, the rise in the corm count rate was due to the formation of a more significant number of shoots at the base of which the daughter corms were formed.

CONCLUSIONS

Using chitosan with a molecular weight of 6000 g · mol^{-1} at a concentration of 0.4% for soaking corms before planting accelerated their emergence only in the cultivars characterised by a long germination period. The biopolymer also accelerated the development of generative organs and extended the flowering period of gladiolus plants, but these effects depended on the cultivar traits and the application method. The impact was more substantial in the cultivars with a long cultivation period and in the case of spraying or watering the plants. Using chitosan significantly increased the plant height and the number of developed leaves. Among the evaluated gladiolus cultivars, Peter Pears was characterised by more intensely green leaves. The decorative value of the cultivated plants only slightly depended on the use of chitosan. The increase in inflorescence shoots and the inflorescence length were observed only in the plants sprayed with chitosan. The number of developed flowers in the inflorescence and their diameter did not differ significantly from plants not treated with chitosan, and these parameters depended mainly on cultivar traits. Soaking the corms before planting and spraying or watering the plants with chitosan increased the weight growth index of daughter corms.

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THE APPEARENCE OF BRANCHED BROOMRAPE (*Phelipanche ramosa* L. Pomel) ON GALLANT SOLDIER (*Galinsoga parviflora* Cav.) AND SOME VEGETABLE CROPS

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SUMMARY

In the pot experiments conducted in the greenhouse and in the open, in the years 2009–2010, 2013–2014, and 2019–2020 in Skierniewice in Poland was found that *Phelipanche ramosa* L. Pomel (branched broomrape) can parasite on the roots of *Galinsoga parviflora* Cav. (gallant soldier) and was also confirmed its ability to parasite some vegetable crops such as Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis* (Lour.) Hanelt.) and the crops from *Apiaceae* family: carrot (*Daucus carota* L. subsp. *sativus* (Hoff.)), celeriac (*Apium graveolens* L. var. *rapaceum* (Mill)) and parsley (*Petroselinum sativum* (Mill) Fuss.). *G. parviflora* is a segetal weed that commonly infests crops in many countries, including Poland. It was found that Chinese cabbage is a better host for *P. ramosa* than *G. parviflora* but the possibility of parasitising *G. parviflora* by *P. ramosa* is new information and it may be a new way to spread this parasite into new areas.

Keywords: *Phelipanche ramosa*, parasitism, *Galinsoga parviflora*, vegetable crops

INTRODUCTION

Phelipanche ramosa L. Pomel (branched broomrape) is a parasite species belonging to *Orobanchaceae* Vent. family. To this family belong over 200 species of parasitic herbaceous plants and from this group, only seven are considered a threat to economically important crops, namely: *Phelipanche aegyptiaca* (Pers.) Pomel (= *Orobanche aegyptiaca* Pers.), *Phelipanche ramosa* L. Pomel (= *Orobanche ramosa* L.), *Orobanche minor* Sm., *Orobanche cernua* Loefl., *Orobanche crenata* Forssk., *Orobanche cumana* Wallr. and *Orobanche foetida* Poir. [Musselman 1980, Kroschel 2001, Rubiales et al. 2009, Parker 2009, Rubiales and Fernández-Aparicio 2012]. *Orobanche* and *Phelipanche* species (the broomrapes) are root parasitic plants, some of which cause heavy yield losses of important crop species [Cimmino et al. 2014].

P. ramosa has the broadest host range, including plants of the families *Solanaceae* (Juss.), *Cannabaceae* (Martinov), *Brassicaceae* (Burnett), and *Fabaceae* (Lindl) [Musselman 1980, Kroschel 2001, Qasem and Foy 2007]. Two broomrape species *O. crenata* and *P. ramosa* attacks the crops of the *Apiaceae* family such a: carrot (*Daucus carota* L. subsp. *sativus* (Hoff.)) celeriac (*Apium graveolens* L. var. *rapaceum* (Mill)), parsley (*Petroselinum sativum* (Mill) Fuss.), fennel (*Foeniculum vulgare* Mill) and of *Fabaceae* family: broad bean (*Vicia faba* L.), pea (*Pisum sativum* L.),

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lentil (*Lens culinaris* Medik.), chick pea (*Cicer arietinum* L.), alfalfa (*Medicago sativa* L.). In many cases *P. aegyptiaca* and *P. ramosa* attack the crops of *Solanaceae* family like tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.) and cause the yield loss of more than 75% [Mauromicale et al. 2008, Hershenhorn et al. 2009].

Some authors found that *P. ramosa* parasite attacks the roots of tobacco [Buschmann et al. 2005a, Karkanis et al. 2007], potato [Haidar et al. 2003, Haidar and Sidahmed 2006], tomato [Mauromicale et al. 2005, 2008, Diaz et al. 2006, Longo et al. 2010, Stępowska et al. 2012, Disciglio et al. 2015, Borkowski et al. 2018], cannabis (*Cannabis sativa* L.) [Buschnann 2004], black medick (*Medicago lupulina* L.) [Musselman and Bolin 2008], cabbage (*Brassica* L.) [Małuszyńska et al. 1998, Piwowarczyk 2012], oilseed rape (*Brassica napus* subsp. *napus*) [Buschmann et.al. 2005b, Moreau et.al. 2016, Yanev et.al. 2020] and other *Brassica*s [Joel et al. 2007]. *P. ramosa*, except potato and tomato is well developing on the roots of red pepper (*Capsicum annuum* L.), eggplant and parasite the roots of some weed species like black nightshade (*Solanum nigrum* L.), henbane (*Hyoscyamus niger* L.) and Jimson weed (*Datura stramonium* L.) [Vouzounis and Americanos 1998, Haidar and Sidahmed 2006]. In Marocco *P. ramosa* infested carrot [Zehhar et al. 2003], in Eastern France celeriac [Gibot-Leclerc et al. 2014] and oilseed rape (*Brassica napus* L. var. *napus*) [Buschmann 2004, Buschmann et al. 2005a, Gibot-Leclerc et al. 2012].

P. ramosa plants parasitising tomatoes produce many slender, erect stems from small bulbs. The yellowish stems grow 10 to 60 cm tall and are coated in glandular hairs. In Poland, the height of *P. ramosa* plants ranged from 5–40 cm [Szafer et al. 1986]. In the experiments carried out on tomatoes at the Research Institute of Vegetable Crops in Poland, in the nineties of the twentieth century, the height of *P. ramosa* shoots was less than 20 cm but in later experiments the height of shoots frequently was over 20 cm [Borkowski et al. 2018]. *P. ramosa* is parasitic on many other plants, draining nutrients from their roots, and it lacks the leaves and chlorophyll. On every big shoot are several flowers, each with yellowish sepals of calyx and with a tubular white and blue to purple corolla of flowers. The life cycle of broomrape plants consists of two stages; a hypogeal (underground) stage and an epigeal (aboveground) stage [Musselman 1980, Lolas 1994, Wegmann 2004]. Broomrapes (*Orobanche* and *Phelipanche* spp.) spend most of their life cycle underground, where undergo processes of germination, haustorial differentiation from the radicle, haustorial penetration of the host, formation of vascular connection with the host, acquisition of host nutrients, and storage of resources in a parasite organ called the tubercle or nodule [Fernández-Aparicio et al. 2011].

Broomrape seeds germinate only in the presence of germination stimulants (sesquiterpene lactones, strigolactones) released by the host plants [Bouwmeester et al. 2003, Cardoso et al. 2010]. After germination they develop a specific attachment organ, described as haustorium, that forms a functional bridge to their hosts. Via haustorium they are able to take up mineral nutrients and assimilates [Dörr and Kollmann 1974, 1975, Fernández-Aparicio et al. 2011]. Because of their limited root system, *Orobanche* and *Phelipanche* species are also dependent on water supply by their host plant. The first step in host-parasitic plant interaction is the stimulation of parasite seed germination by compounds released from host roots. According to the host, the duration of its life cycle can range from 12 weeks (tomato/tobacco) to 40 weeks (oilseed rape). It was found that in tobacco *P. ramosa* plants emerge above ground after an underground shoot development at approximately 45 to 55 days (in warm climates), flowers 4 to 5 days later, and the tiny seeds (0.3 mm in diameter, approx. 100.000 per plant) ripen after another 20 to 25 days [Lolas 1994, Wegmann 2004]. However, this can vary according to soil types and transplanting dates. This provides the parasite with great genetic adaptability to environmental changes, including host resistance, agronomical practices, and herbicide treatments [Joel et al. 2007]. In the experiment with tomatoes, conducted in Poland, it was proved that *P. ramosa* shoots emerges above ground after 62 days or later [Borkowski and Dyki 2008].

P. ramosa is an important problem in some cultivation areas. *Phelipanche* is a parasite genus that needs a warm climate for growth and development. Progressive variability of climate on the whole world may cause that *P. ramosa* will be an increasing problem in many countries. Now the climate in Poland is get-

ting warmer and *P. ramosa* would be more dangerous for cultivated crops [Dyki et al. 2015, Piwowarczyk 2012]. In Poland *P. ramosa* belongs to the group of rare species, potentially endangered, and during the years 2004–2014 was under strict protection and from 2014 it is under partial protection [Dz.U. 2014, poz. 1409].

The management of *P. ramosa* infestation and control depends on its growth stage and method of control. In solarised soil, no broomrape shoots emerged, and neither haustoria nor underground tubercles of the parasite were found on tomato roots [Mauromicale et al. 2005]. The development of herbicides based on natural metabolites from microbial and plant origin, targeting early stages of parasitic plant development, might contribute to the reduction of broomrape seed bank in agricultural soils.

Galinsoga parviflora (Cav.) (gallant soldier) is an herbaceous plant that descended from South America and is very common in Poland [Ławrynowicz and Warcholińska 1992] and in other European countries. It is an annual dicotyledonous weed species in many countries and is one of the most troublesome weeds in vegetable crops in Poland [Dobrzański 1996]. It grows most often in cultivation fields, but can also be found on roadsides, streets and dumpsters, both in the shade and sunlight places [Parylak 1988]. *G. parviflora* has a short growing season, emerges from the spring to autumn, grows very quickly, blooms 4–6 weeks after emergence, and in one season can give even 2–3 generations. The seeds can germinate immediately after falling onto the soil surface. Except for *G. parviflora* the common species observed on the field is hairy galinsoga (Galinsoga quadriradiata Ruiz & Pav = *Galinsoga ciliata* (Raf.) S. F. Blake).

The studies on the development and control of *P. ramosa* were carried out over 10 years at the National Institute of Horticulture Research (formerly the Research Institute of Vegetable Crops) [Borkowski et al. 2007, 2018, Dyki et al. 2009, Stępowska et al. 2011, 2012]. The experiments mainly concerned the parasitising of *P. ramosa* on tomato plants. In the studies on *P. ramosa* development parasitising tomato plants, light and scanning electron microscopy was used [Stępowska and Dyki 2012].

The studies aimed to determine in Polish conditions the parasitising ability of *P. ramosa* on the roots of *G. parviflora*, Chinese cabbage and vegetable crops from *Apiaceae* family: carrot, celeriac, and parsley. These species are the host plants of *P. ramosa*.

MATERIAL AND METHODS

The first observations on *Phelipanche ramosa* parasitise on *Galinsoga parviflora* roots

The field experiment on the growth regulators assessment in tomatoes was performed in 2008 in Skierniewice in Poland. The experiment was established in the place, where earlier the tests with *P. ramosa* control on tomatoes were carried out. After finishing the experiment, the plants of tomatoes were removed and the plots were left for some weeks without any treatments. Many weeds were left on the plots, among which the *Galinsoga parviflora* was the dominant one. During the hand weeding of these plots, small shoots of *P. ramosa* were visible and disappeared some days after weeding. It aroused the suspicion, that the roots of another host could be in the soil and it could be *Galinsoga parviflora*. The observation made in 2008 was the basis for undertaking the studies in the next years on *P. ramosa* development on the roots of *G. parviflora*.

The observations on the appearance of *Phelipanche ramosa* on Chinese cabbage and *Galinsoga parviflora* roots

The observations on the appearance of *P. ramosa* on Chinese cabbage and *G. parviflora* were made in the years 2009–2010 in Skierniewice in Poland, during the studies on the prevention of tipburn on Chinese cabbage, using calcium nitrate and biostimulants. In the experiments the seeds of Chinese cabbage cv. Bilko F_1, resistant to the clubroot (*Plasmodiophora Brassicae*) and fusariosis (*Fusarium oxysporum*), were sown in the greenhouse to multipods on March 28th in 2009 and April 8th in 2010 and the young seedlings of Chinese cabbage, having 3–4 true leaves, were planted in the open into the small containers (20 × 20 cm) on April 27th in 2009 and on May 7th in 2010. In 2009 the pods were filled up with the substrate, in which earlier the tomatoes parasitised by *P. ramosa* were grown, while in 2010 were filled up with peat substrate mixed with *P. ramosa* seeds. The experiment was set up in 11 replications. The containers were kept in the open until the end of the experiment.

The observations on tipburn on Chinese cabbage were completed at the beginning of July, and the healthy plants of Chinese cabbage were harvested, and small, rotted, and injured plants remained in containers, and were kept 3 months longer to make further observations. In the middle of July less than 30% of Chinese cabbage plants, remaining in containers, were alive and some various weed species, mainly *G. parviflora*, occurred in all containers. The seedlings of *G. parviflora* accounted for over 60% of the weed population. The Chinese cabbage plants have been removed from the half of containers and the weeds, except *G. parviflora*, were removed from all containers. *G. parviflora* occurred in containers with and without Chinese cabbage. The plants of *G. parviflora* were trimmed by 50%, to stimulate their regrowing. During the experiments, the dates of *P. ramosa* sprouting, the height of *P. ramosa* plants, flowering and forming capsules by *P. ramosa* were observed. The observations of *P. ramosa* shoots were carried out till October.

The results were statistically analysed by analysis of variance using Statistica Program v. 13.0 (Statsoft Inc.). The Newman-Keuls test (p = 0.05) was used to compare the significance of the means.

The studies on the appearance of *Phelipanche ramosa* on *Galinsoga parviflora*

The experiments aimed to confirm the parasite of *G. parviflora* by *P. ramosa*. The greenhouse experiments were conducted in the years 2013–2014 at the Research Institute of Vegetable Crops in Skierniewice. The experiment was conducted in 5 replications, in 5-liter pots filled up with the substrate made of pseudo-podzolic soil (75%) mixed with peat (25%) and fertilizers. The seeds of *G. parviflora* were sown into all pods, Chinese cabbage to 1/3 amount of pots, and the seeds of *P. ramosa* were sown into the pots with *G. parviflora* alone and pots sown with Chinese cabbage and *G. parviflora* together. The control was the pots with *G. parviflora* alone. The seeds of *P. ramosa* were mixed with substrate before filling the pots. The source of seeds was an earlier experiment on the appearance of *P. ramosa* on tomatoes, carried out in a previous year. To each pot 10 seeds of Chinese cabbage, 12 seeds of *G. parviflora*, and about 200 seeds of *P. ramosa* were sown. The pots were filled up with the substrate and the seeds of *G. parviflora* and Chinese cabbage were sown about 2 cm deep into the soil. The seeds were sown on May 15th, 2013, and on June 24th, 2014. After emergence, Chinese cabbage was thinned to 6 plants per pot and *Galinsoga parviflora* to 8 plants per pot. During the experiment, Chinese cabbage plants were fertilized with the liquid fertilizer Novokont, containing the macro and microelements.

After 2 months of vegetation *G. parviflora* plants were rotten and most of the leaves had dried up, so the upper parts of the plants were cut and the above parts of plants having 10 cm height were left. The plants of *G. parviflora* were cut 93–95 days after sowing the seeds because at that time, appear any shoots of *P. ramosa*. The aim of cutting was to stimulate the regrowing of this weed and keep it a longer time to give the possibility for germination of *P. ramosa* seeds, shoots emergence and growth, and flowering. *G. parviflora* were not removed from the pots throughout the season. The observation of *P. ramosa* germination was performed systematically until the end of October.

Preliminary studies on the appearance of *P. ramosa* on carrot, parsley, and celeriac

In 2019–2020 the pot experiments on the appearance of *P. ramosa* on the roots of three vegetable crop species from the *Apiaceae* family: carrot (*Daucus carota* L.), cv. Nerac F1, celeriac (*Apium graveolens* L. var. *rapaceum* (Mill)), cv. Maxim and parsley (*Petroselinum sativum* (Mill) Fuss.) cv. Eagle was carried out. The pots were filled up with pseudo-podzolic soil (75%) mixed with peat (25%) and for each crop 5 pots with *P. ramosa* seeds and 5 pots without *P. ramosa* seeds were prepared. For every pot 8 transplants of celeriac were planted and in the case of carrot and parsley 12 seeds per 1 pot were sown. The experiments were performed in 5 replications and the seeds were sown on June 8th, 2019 and, May 15th, 2020. One month after sowing/transplanting (July 10th, 2019, and June 14th, 2020) in all pots the young seedlings were thinned to 6 plants per pot.

In both years all pots were fertilized 7 times with foliar fertilizer Novocont and mixed fertilizers Azofoska and Rosasol. The pests were controlled according to official recommendations: aphids (*Aphis gossypii*) by lambda-cyhalothrin, spirotetramat and deltamethrin and powdery mildew (*Erysiphe cichora-*

ceum) by boscalid + pyraclostrobin and fluopyram + tebukonazol mixtures.

The observations on *P. ramosa* shoot sprouting in 2020 were carried out until December 30th. The winters of 2019/2020 and 2020/2021 were not frosty, so in such conditions, it was possible to continue the experiments in a cold greenhouse and the open until June. The observations were made during all vegetation periods. At finishing the experiments on every shoot of *P. ramosa* the bags with seeds were counted.

The morphological analyses of plants and their roots

In the pot experiments carried out in 2013–2014, the macroscopic evaluation of *G. parviflora* and *P. ramosa* plants was done and then the samples of plant material were collected for microscopic analyses. *G. parviflora* and *P. ramosa* plants were removed from pots together with the substrate and the soil was washed out carefully to keep the clean roots alone. The tight connections between the roots of *G. parviflora* and *P. ramosa* were observed in the root mass. The morphological studies on *P. ramosa* and *G. parviflora* roots and their structural connections were carried out using a stereomicroscope (STM) Olympus SZX 16 with a Cell B program to prepare photographic documentation. The photos of the characteristic root connection between *G. parviflora* and *P. ramosa* were done and presented at the end of this paper.

RESULTS AND DISCUSSION

The first observations on *Phelipanche ramosa* parasitising *Galinsoga parviflora*

The first observation suggesting that *P. ramosa* can parasitise *G. parviflora* was obtained in 2008, in a field experiment assessing the growth regulators in tomatoes [Borkowski et al. 2007, Borkowski and Dyki 2008], where at the end of the experiment the various weed species were observed on the plots. The number of *G. parviflora* plants exceeded 50% of the total weeds number in the weed population. After finishing the experiment, tomato plants were removed, and the plots were left for some weeks without any treatments. During the hand weeding of the plots, small shoots of *P. ramosa* were visible, and they were lost some days after weeding. The removal of tomato plants with roots parasitised by *P. ramosa* did not cause the death of parasite plants, what raised the suspicion that the origins roots of another host could be in the soil. *G. parviflora* was considered a possible host species. The source of *P. ramosa* was the soil, in which in previous years the experiments with *P. ramosa* control on tomatoes were carried out.

The observations on the appearance of *Phelipanche ramosa* on Chinese cabbage and *Galinsoga parviflora*

In the experiment conducted in the greenhouse and in the open, the sprouting shoots of *P. ramosa* near Chinese cabbage plants and in the pots with *G. parviflora*, without cabbage, were noted. In 2009 the first shoots of *P. ramosa* were found in 3 containers with Chinese cabbage on August 2nd (97 DAP) and on August 17th (112 DAP) the shoots were visible in 11 containers. The sprouting of *P. ramosa* shoots in those containers was observed until the end of September. Some shoots flowered (Tab. 1) but rarely formed capsules with seeds because the shoots quickly dried up. The maximum height of shoots in all containers was 5 cm on August 2nd and 6 cm on August 17th. Borkowski and Dyki [2008] reported that in earlier stud-

Table 1. The terms of sprouting and development of *P. ramosa* in experiments (Skierniewice, 2009–2010)

Year	Planting of Chinese cabbage	Sprouting of *P. ramosa* (first shoot)		Containers with *P. ramosa*	Height of *P. ramosa* plants (cm)	*P. ramosa* flowering	Forming capsules by *P. ramosa*
		Date	DAP				
2009	27.04	2.08	97	3	5	yes	no
		17.08	112	11	6	yes	no
2010	7.05	17.08	102	1	13	yes	yes
		31.08	116	6	15	yes	yes

DAP – days after planting

Fig. 1. A shoot of *P. ramosa*, close to a flowering and seed-forming *G. parviflora* plant

ies with tomatoes, conducted in a greenhouse, the first shoots of *P. ramosa* appeared 62 days after planting. The faster emergence of *P. ramosa* was caused by better growth conditions in the greenhouse, mainly higher temperatures.

In 2010 the first shoot of *P. ramosa* was visible on August 17th (102 DAP of Chinese cabbage) in containers where the Chinese cabbage was completely rotten and died, and only the plants of *G. parviflora* remained. *P. ramosa* plants were visible close to *G. parviflora* plants. Then the shoot flowered and later formed capsules (Tab. 1). At 116 DAP, the shoots of *P. ramosa* were observed in 6 containers. In macro and microscopic analysis it was found that the roots of *G. parviflora* were connected with the parasite plant (Fig. 2, 3). It confirms that *G. parviflora* is a new host of *P. ramosa*. In the experiment *P. ramosa* shoots were not higher than 13 cm (Tab. 2). In earlier studies with tomatoes [Borkowski et al. 2018] *P. ramosa* plants were even 29 cm high. It suggests that the tomato is a better host for *P. ramosa* than Chinese cabbage and *G. parviflora*. The observations confirmed the earlier assumption that *P. ramosa* may parasitise on the roots of *G. parviflora*. The preference of *P. ramosa* to parasitise the various plants can change in the future, mainly due to climate change. Gibot-Leclerc et al. [2012] report that over 30 years ago *P. ramosa* was not a problem in oilseed rape in France but now poses a threat.

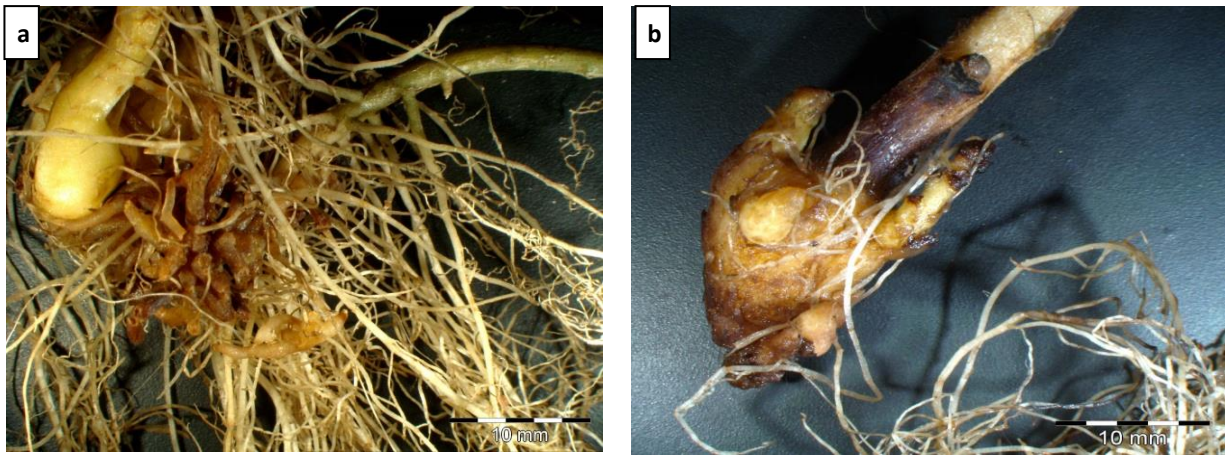

Fig. 2. The dark roots of *P. ramosa* overgrown the *G. parviflora* roots

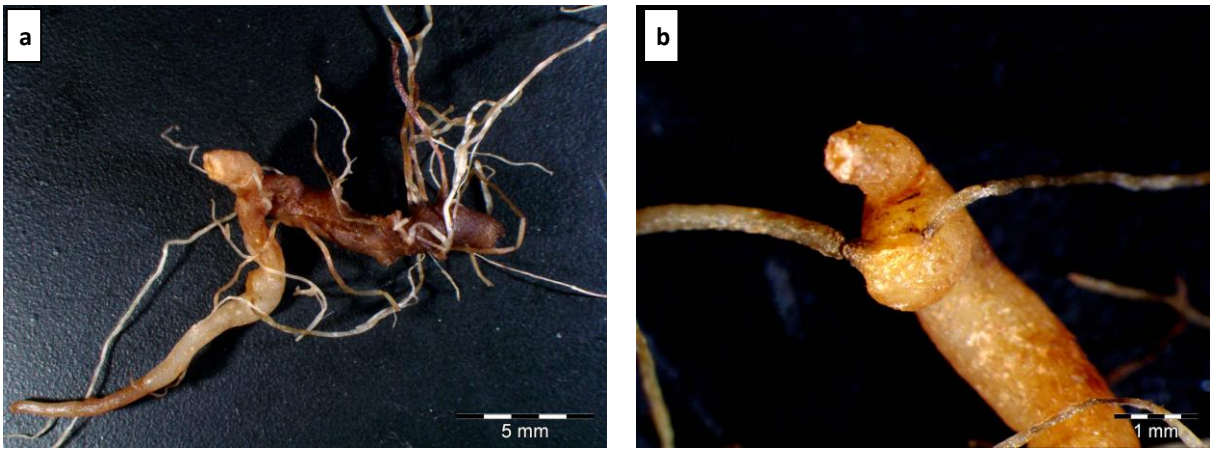

Fig. 3. The roots of *Galinsoga parviflora* connected with parasite *Phelipanche ramosa*

The studies on the appearance of *Phelipanche ramosa* on *Galinsoga parviflora*

In the greenhouse experiments conducted in 2013–2014, the seeds of *P. ramosa* were mixed with the substrate used for filling the pots with *G. parviflora* alone and *G. parviflora* with Chinese cabbage. The check was the pots with *G. parviflora* alone (Tab. 2). During vegetation, *P. ramosa* shoots were visible in the pots containing *G. parviflora* plants and also *G. parviflora* with Chinese cabbage. The first shoot of *P. ramosa* appeared 120–125 days after sowing the seeds.

The average height of *P. ramosa* shoots grown in the pots with *G. parviflora* and Chinese cabbage amounted to 4.2 cm (Tab. 2) and was higher than in the pots with *G. parviflora* alone (2.8 cm height). The highest shoot of *P. ramosa*, at 173–176 DAS was obtained in the pot with Chinese cabbage and *G. parviflora* and was 10 cm at the first observation, and 13 cm at the second observation (180–182 DAS). In the pots with *G. parviflora* alone, the highest shoot was lower in both terms of observations and resulted in 7 and 12 cm, respectively. It shows that in the pots with Chinese cabbage and *G. parviflora*, the growth of *P. ramosa* was faster than in the pots with *G. parviflora* alone, which suggests that Chinese cabbage is a better host for *P. ramosa* than *G. parviflora*.

The results obtained at 154–155 DAS showed that the number of *P. ramosa* shoots in the pots with

Table 2. The height of *Phelipanche ramosa* shoots in pot experiments (Skierniewice, 2013–2014)

Object	The average height of *P. ramosa* shoots per 1 pot (cm)					The average height (cm)	The height of the highest shoot (cm)	
	DAS							
	180–182					–	173–176	180–182
	I	II	III	IV	V	–	–	–
G. parviflora (check)	0	0	0	0	0	0	0	0
G. parviflora + *P. ramosa*	3.9	1.6	2.9	3.3	2.2	2.8 b	7.0 b	12.0 a
G. parviflora + Chinese cabbage + *P. ramosa*	4.5	1.8	6.2	3.0	5.5	4.2 a	10.0 a	13.0 a

DAS – days after sowing
I – V – replications

Table 3. Characteristics of *P. ramosa* plants occurring on *G. parviflora* and Chinese cabbage (Skierniewice, 2013–2014)

Object	The number of *P ramosa* shoots per pot			Shoots with flowers (%)	Shoots with capsules per pot
	DAS				
	154–155	173–176	180–182	180–182	188–190
G. parviflora – check	0	0	0	0	0
G. parviflora + *P. ramosa*	4.3 b	7.7 b	7.9 b	14.7 b	0.6 a
G. parviflora + Chinese cabbage + *P. ramosa*	8.6 a	20.2 a	20.4 a	25.5 a	0.7 a

DAS – days after sowing. The first shoot of *P. ramosa* appeared 120–125 DAS

G. parviflora and Chinese cabbage growing together (8.6 shoots per pot) was two times higher, in comparison to the pots with *G. parviflora* alone (4.3 shoots). On subsequent assessment terms, the differences were even greater. At 173–176 DAS the number of *P. ramosa* shoots increased to 20,2 and 7.7 shoots per pot, and at 180–182 up to 20.4 and 7.9 shoots, respectively.

The tallest shoots of *P. ramosa* were obtained in the pots containing *G. parviflora* with Chinese cabbage (Tab. 2) and 25.5% of shoots from these pots produced flowers, while from the pots with *G. parviflora* alone, only 14.7% (Tab. 3). The shoots with capsules were similar in both objects (0.7 and 0.6 shoots per pot).

It was noticed faster germination and better growth of *P. ramosa* in the pots with *G. parviflora* and Chinese cabbage, in comparison to *G. parviflora* alone (Tab. 3). The greater number of shoots per pot (Tab. 3) and the highest shoot of *P. ramosa* (Tab. 2) were obtained in the pots with *G. parviflora* and Chinese cabbage and the shoots of *P. ramosa* from these pots produced more flowers (Tab. 3).

The results confirm that Chinese cabbage is a better host plant for *P. ramosa* than *G. parviflora*. It should be noted that the number of *P. ramosa* shoots produced and their height depended on the host plant species, while the production of flowers and the formation of capsules depended mainly on the stage of the development cycle.

Preliminary studies on the appearance of *P. ramosa* on carrot, parsley, and celeriac

In the experiments conducted in Poland, it was found that *P. ramosa* can parasitise on carrot, celeriac, and parsley roots. *P. ramosa* is a root-holoparasitic angiosperm plant, which is a pest in agricultural fields, infesting a wide range of crop species [Parker and Riches 1993]. This species is known as a parasite plant almost the whole world [Zehhar et al. 2003, Buschmann 2004, Diaz et al. 2006, Joel et al. 2007, Borkowski and Dyki 2008, Piwowarczyk 2012, Dyki et al. 2015] and it has the serious menace for cultivated crops, especially in countries with warm climate.

In the greenhouse experiment carried out in 2019/2020, the shoots of *P. ramosa* for the first time were observed 128 DAS (October 14th, 2019) in one pot with carrot, 142 DAS (October 28th, 2019) in two pots with celeriac, and 205 DAS (December 30th, 2019) in one pot with parsley (Tab. 4). At the end of the year (205 DAS), the shoots were observed in 2 pots with carrots, 4 pots with celeriac and 1 pot with parsley. After winter time, at 299 DAS (April 3rd, 2020) the shoots of *P. ramosa* were visible in 2 pots with carrot, 4 with celeriac and 5 pots with parsley. The results show a different ability of *P. ramosa* to connect with the roots of vegetable crops from the *Apiaceae* family, and do not always occur the infestation.

The average number of *P. ramosa* shoots per 1 pot on April 3rd, 2020 (299 DAS) was the highest in the pots with celeriac (19.2) lower in parsley (6.8) and the lowest in carrot (5.2), while at the end of 2019, the number was 8.7, 2.2 and 1.2 shoots per 1 pot, respectively. It indicates that *P. ramosa* systematically forms connections with vegetable crop roots (Tab. 4). The results show that the emergence period and the growth dynamics of *P. ramosa* vary, depending on the host plant. The earliest emergence of *P. ramosa* was

observed in the pots with carrots and the latest in the pots with parsley. The growth dynamic of *P. ramosa* was the highest in the pots with celeriac.

The height of *P. ramosa* shoots in carrot, parsley, and celeriac at 236 DAS (on January 30th, 2020) ranged between 10 and 13 cm, and at 299 DAS (on April 3rd) between 11 and 16 cm (Tab. 5). On January 30th, 2020 (236 DAS) the highest plants of *P. ramosa* were obtained in pots with celeriac (13 cm), while on April 3rd (299 DAS) with parsley (16 cm). It shows that *P. ramosa* grows well in celeriac and parsley and slightly slower in carrots. *P. ramosa* produced capsules with seeds in all objects. The most capsules were produced in the pots with parsley (116.8) and the least in pots with carrots (34.2 capsules). The capacity of capsule production is important for the spread of this parasite.

In the growing season of 2020/2021, the first shoot of *P. ramosa* was visible at 110 DAS (September 2nd, 2020) in one pot with carrot, 117 DAS (September 9th, 2020) in three pots with parsley, and 165 DAS (October 27th, 2020) in one pot with celeriac. On October 27th the shoots of *P. ramosa* were visible in 4 pots with carrots, 3 pots with parsley, and 1 pot with celeriac,

Table 4. The appearance of *P. ramosa* in vegetable crops from *Apiaceae* family (Skierniewice, 2019/2020)

Crop	Number of pots with *P. ramosa* shoots				Number of *P. ramosa* shoots per 1 pot	
	DAS					
	128	142	205	299	205	299
Carrot	1	1	2	2	2.2 b	5.2 b
Celeriac	0	2	4	4	8.7 a	19.2 a
Parsley	0	0	1	5	1.2 b	6.8 b

DAS – days after sowing. Dates of evaluation: 128 DAS – 14.10.2019; 142 DAS – 28.10.2019; 205 DAS – 30.12.2019; 299 DAS – 3.04.2020

Table 5. The appearance of *P. ramosa* in vegetable crops from *Apiaceae* family (Skierniewice, 2019/2020)

Crop	The average height of *P. ramosa* shoots (cm)				Number of capsules with seeds in 1 pot
	DAS				
	142	205	236	299	360
Carrot	7.2 a	9.1 ab	10 b	11 b	34.2 c
Celeriac	9.4 a	13.2 a	13 a	15 a	88.0 b
Parsley	0	4.8 b	10 b	16 a	116.8 a

DAS – days after sowing. Dates of evaluation: 142 DAS – 28.10.2019; 205 DAS – 30.12.2019; 236 DAS – 30.01.2020; 299 DAS – 3.04.2020; 360 DAS – 5.06.2020

Table 6. The appearance of *P. ramosa* in vegetables crops from *Apiaceae* family (Skierniewice, 2020/2021)

Crop	Number of pots with *P. ramosa* shoots					Number of *P. ramosa* shoots per 1 pot		
	DAS							
	110	117	165	195	346	195	229	357
Carrot	1	2	4	4	5	2.0 a	2.0 b	2.6 b
Celeriac	0	0	1	5	5	2.8 a	4.2 a	15.6 a
Parsley	0	3	3	3	4	2.2 a	2.2 ab	3.7 b

DAS – days after sowing. Dates of evaluation: 110 DAS – 2.09.2020; 117 DAS – 9.09.2020; 165 DAS – 27.10.2020; 195 DAS – 26.11.2020; 229 DAS – 30.12.2020; 346 DAS – 26.04.2021; 357 DAS – 7.05.2021

while at 346 DAS (April 26th, 2021), the shoots were observed in 5 pots with carrots and celeriac and 4 pots with parsley (Tab. 6). The number of shoots per 1 pot on November 26th, 2020 (195 DAS) ranged from 2.0 to 2.8 and on December 30th, 2020 (229 DAS) from 2.0 to 4.2. In the next year, on May 7th (357 DAS) the highest number of shoots was noted in celeriac (15.6), while much lower in carrot and parsley (2.6 and 3.7 shoots).

The smallest shoots of *P. ramosa*, in all terms of observations, were obtained in the pots with carrots, and the highest in the pots with parsley. On December 30th (229 DAS) the average height of *P. ramosa* shoots in carrots was 9 cm, in celeriac 11 cm, and in parsley 14 cm (Tab. 7). In the next year, on April 26th, 2021 (346 DAS) the average height of *P. ramosa* shoots was 8 cm in the pots with carrots, 10 cm in the pots with celeriac, and 11 cm in the pots with parsley. After winter the height of *P. ramosa* shoots was lower than before winter because the highest shoots died at low temperatures and remained only younger, lower plants.

The capsules with seeds were observed on *P. ramosa* shoots in all objects. The seeds of the crops grown in the test, and *P. ramosa* were sown in the year of 2020 and capsules of *P. ramosa* were counted in 2021. The highest number of capsules with seeds was obtained in the pots with celeriac and the lowest in the pots with carrots. In the pots with celeriac 291.6 capsules were obtained, in the pots with parsley 14.2 and in the pots with carrot 12.6 (Tab. 7).

The results of experiments show that *P. ramosa* can parasite carrot, celeriac, and parsley, and the plants of celeriac seem to be a better host for this parasite than carrot and parsley. *P. ramosa* may be harmful to all these crops. The results of the studies confirm previous reports of some authors on the occurrence of *P. ramosa* in these species. However, those reports mainly concern the occurrence of *P. ramosa* in South European countries. Joel et al. [2007] maintain that *P. ramosa* can damage parsley, celery, parsnip, lettuce, melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunb.) and cucumber (*Cucumis sativus*), similarly to *P. aegyptiaca*. Gibot-Leclerc et al. [2014] have reported that celeriac on clay soil in the Champagne--Ardennes region in Eastern France was infested by *P. ramosa* where this parasite had been observed 4 years before. The negative symptoms like slower growth of celeriac, chlorosis along the leaves, and decreasing the yield were observed on host plants. The infestation of celeriac was confirmed by verifying the attachment of *P. ramosa* to celeriac roots. Gibot--Leclerc et al. [2012] have reported that in France *P. ramosa* can decrease the yield of oilseed rape up to 90%. Nowadays, it is a problem but 30 years ago *P. ramosa* was not parasite oilseed rape at all.

The morphological analyses of plants and their roots

The plants of *G. parviflora* and *P. ramosa* collected from the experiments were analysed morphologically, their roots removed from the pots were washed out carefully, to remain the clean roots alone, and subjected to microscopic analysis. Microscopic analysis showed a tight connection between the roots of *G. parviflora* and *P. ramosa*, observed in the root mass. It confirms that *P. ramosa* may parasitise on the roots of *G. parviflora*. The tight connections are a specific organ called haustorium, which is used to take up mineral nutrients and assimilates them [Dörr and Kollmann 1974, 1975, Fernández-Aparicio et al. 2011]. The photos

Table 7. The appearance of *P. ramosa* in vegetables crops from *Apiaceae* family (Skierniewice, 2020/2021)

Crop	The average height of *P. ramosa* shoots (cm)			Number of capsules with seeds in 1 pot
	DAS			
	195	229	346	383
Carrot	9 b	9 b	8 a	12.6 b
Celeriac	11 ab	11 ab	10 a	291.6 a
Parsley	13 a	14 a	11 a	14.2 b

DAS – days after sowing. Dates of evaluation: 195 DAS – 26.11.2020; 229 DAS – 30.12.2020; 346 DAS – 26.04.2021; 383 DAS – 2.06.2021

of connections between *G. parviflora* and *P. ramosa* were placed in the next of this work. The descriptions of *P. ramosa* plants and flowers with colored photos were also presented by Borkowski et. al [2018], Dyki and Borkowski [2016], and Stępowska et al. [2012]. The conclusion on the appearance of *P. ramosa* on *G. parviflora* roots is entirely new information, not found earlier in literature.

CONCLUSION

Studies have shown that *Phelipancha ramosa* can parasitise the roots of *Galinsoga parviflora*, Chinese cabbage, and some vegetable crops from the *Apiaceae* family: carrot, parsley, and celeriac. The Chinese cabbage and *G. parvifllora* have a short growing season, which in Poland may be unfavourable for the full development of *P. ramosa* and seed production. It should be assumed that in the absence of the host, *P. ramosa* can spread from Chinese cabbage to *G. parviflora* plants, so not plowing the soil after Chinese cabbage harvest may cause the transmission of *P. ramosa* on *G. parviflora* roots, its further growth and production the seeds.

Galinsoga parviflora commonly appears in Poland and many other countries, so the knowledge of the possibility of parasitising the roots of *G. parviflora* by *P. ramosa* is important, because this parasite may contribute to the spread of *P. ramosa* to new areas. *P. ramosa* is a thermophilic plant parasite and requires a higher daily temperature to germinate, so occurs mostly in warm climate countries. Without the host, this parasite does not develop at all. Due to global climate changes, the area where *P. ramosa* occurs is constantly increasing, so in the future can also become a threat to cultivated crops in Poland. It should be emphasized that in case of *P. ramosa* occurrence on the field, *G. parviflora* must be effectively controlled.

The information on *P. ramosa* parasitising the roots of *G. parviflora* is completely new and is not available in the literature.

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CALLUS INDUCTION AND ESTABLISHMENT OF EFFICIENT REGENERATION SYSTEM FOR A NEWLY DEVELOPED LINE OF *Ananas comosus* (L.) MERRILL

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ABSTRACT

In vitro culture method is developed for propagation of a new line of *Ananas comosus* (L.) Merrill selected from a spontaneous mutant of cultivar Yellow Mauritius. The stem with leaves base obtained from sucker buds was selected as explants. The effects of type and concentration of different plant growth regulators on callus induction, adventitious bud formation and plant regeneration were investigated by the single factor, complete combination and L_9 (3^4) orthogonal experiment. A large number of adventitious buds differentiated on Murashige and Skoog (MS) medium with 4.0 mg·L^{-1} 6-BA, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} KT, reaching differentiation coefficient over 12.8. Browning of callus after 2–3 subcultures was eliminated by the application of 1.0 g·L^{-1} of activated carbon in the optimal medium, which didn't significantly reduce differentiation coefficient. The main shoots in adventitious buds were higher in number and stronger suitable for rooting in culture. Hundred percent rooting *in vitro* was achieved on half-strength MS medium with 1.0 mg·L^{-1} NAA. The survival rate of the tissue culture-raised plants was 100%. The methods developed and defined can be used not only for improved the yield of *in vitro* plants, but also for shortening the whole culture cycle.

Keywords: activated carbon, adventitious bud, browning of callus, *in vitro*, rapid propagation

INTRODUCTION

Ananas comosus (L.) Merrill, a perennial herb of genus *Ananas* in Bromeliaceae, is commonly called pineapple. It is one of the four famous fruits in the tropics and subtropics [Editorial Board of Flora of China 1997]. Pineapple is not only delightful in colour, aroma and taste, but also rich in nutrition. Among them, vitamin C content is 5 times that of the apple. In addition, it is abundant in proteinase, which can help the human body digest protein [Ali et al. 2020]. Therefore, it has a broad planting prospect.

Pineapple seedlings are mainly bred by asexual reproduction, with occasional sexual reproduction. Sexual reproduction is performed with artificial assistance. Plant growth is usually slow and the first sexual cycle taking about 24 months in tropical conditions. In addition, due to the high heterozygosity of most parent varieties, most important traits are highly isolated in young plants and the selection cycle is longer, which makes them unsuitable for direct commercial cultivation [Reinhardt et al. 2018]. Traditionally, pineapple propagated asexually by various buds (buds derived from slips, hapas, suckers and crowns) obtained from the parent plant. On average, 2–3 propagules can be produced every year, and it takes 30 years to get

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10,000 m^2 of planting material from one plant, which cannot satisfy the growing demand for planting. Also, diseases which caused by bacteria and viruses carried by the mother strain continued to be spread in the propagules, thus posing serious problems for production [Sastry and Sastry 2013]. Moreover, due to vegetative propagation, most varieties of pineapple have strong self-compatibility, so it is very difficult to develop new varieties by traditional breeding methods. Pineapple is one of the few crops in which all cultivated varieties are derived from a spontaneous variant and natural evolution [Osei-Kofi et al. 1997].

Tissue culture technique is considered a better choice for breeding variant plants with excellent characters [Reinhardt et al. 2018]. Through tissue culture technology, not only the original excellent traits can be retained, but also the excellent single plant can be rapidly propagated into clones, and then a large number of promotions in the production, providing an experimental basis for molecular breeding and genetic engineering. Although there have been many reports on pineapple tissue culture [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Ibrahim et al. 2013], and many attempts have been made on various automated liquid culture systems for micropropagation [Escalona et al. 1999], the expanded application of tissue culture still needs to improve the regeneration coefficient and acclimation scheme [Reinhardt et al. 2018]. A high regeneration efficiency of pineapple, although expressed by bud aggregation in the existing reports [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Mendonça et al. 2017, Cacaï et al. 2023, Lakho et al. 2023, Torres Ruiz et al. 2023], the obtained adventitious buds were weak without regenerated plant morphology, which requires repeated rejuvenation culture [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Reinhardt et al. 2018].

In this study, we removed the previous idea of inducing bud aggregation, and the plants of spontaneous mutant derived from *A. comosus* Yellow Mauritius were used as explants. The initial culture, callus induction, adventitious bud occurrence and proliferation, rooting *in vitro*, acclimation and field transplanting for pineapple were systematically studied. An efficient and stable artificial propagation technology system for a newly developed line of *A. comosus* was established. Although the proliferation coefficient was slightly lower than that reported by bud aggregation on the surface, the efficiency of propagation and plant formation was much higher than that reported by others. The results of this study can provide a theoretical basis and technical support for rapid propagation and industrial production of the plant *in vitro* of pineapple. Meanwhile, it can also provide experimental reference for other varieties of pineapple and artificial rapid propagation of variants with desirable characters.

MATERIALS AND METHODS

Plant materials and establishment of aseptic system

In May 2017, the variant of *Ananas comosus* (L.) Merrill plants were obtained from Longfei farm in Xishuangbanbanna, Yunnan, China (100°49'E, 22°01'N, 950–1,000 m a.s.l.). 30 sucker buds were selected as explants and returned to the laboratory for treatment within 48 h. These explants were washed with running water, and then the dirt on the surface and leaf axils was washed thoroughly with 10% washing powder solution (w/v). To facilitate further sterilization, the leaves of sucker bud were removed, leaving only the stem with 4–5 cm leaves base. Subsequently, they were transported into a sterile operating platform where they were treated with 75% ethanol solution (v/v) for 15 s, and then disinfected with 0.1% $HgCl_2$ (w/v) for 8, 10, 12, 14, 16, and 18 min. After that, each stem with leaves base was washed with sterile water for 6 times, each time no less than 3 min. The surface moisture was dried using a sterilized filter paper. Finally, the base of the leaf was removed by another 1 cm and then 3–4 cm stem with leaves base was used for initial culture.

Medium

Basic medium. Basic medium for all stages was Murashige and Skoog (MS) [Murashige and Skoog 1962] (1/2MS medium for rooting culture) with 3% sucrose (w/v) and 0.55% agar (w/v). Different concentrations of 6-benzylaminopurine (6-BA), zeatin (ZT), kinetin (KT), α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and α-naphthaleneacetic acid (NAA) were added according to

the actual requirement. The reagents and plant growth regulators (PGRs) used in the experiment were purchased from Dingguo Biotechnology (Beijing, China). The pH of the medium was adjusted to 5.6–5.8 with 1 N HCl before autoclaving (121°C, 22 min).

Initial medium. Due to the limited number of explants, it was not possible to use a variety of initial medium for experiments. Therefore, MS medium with 1.5 mg·L^{-1} ZT was used as the initial medium for the stem with leaves base culture. After the successful establishment of an aseptic system, the number of sterile shoots was insufficient for single factor experiment, and the explant accumulation culture was continued in MS medium with 1.5 mg·L^{-1} ZT. Single factor experiment was carried out after 3–5 subcultures in the initial medium.

Single factor medium. Different concentrations of 6-BA (1.0, 2.0, 3.0, and 4.0 mg·L^{-1}), KT (0.5, 1.0, and 2.0 mg·L^{-1}), 2, 4-D (0.01, 0.05, and 0.1 mg·L^{-1}) and NAA (0.5, 1.0, 1.5, and 2.0 mg·L^{-1}) were added to the basal medium, respectively. After 40 days, the growth status of each treatment was observed and analysed to screen out the suitable PGRs types and concentrations.

Medium for callus induction. A complete combination experiment was conducted with 6-BA (2.0, 3.0 and 4.0 mg·L^{-1}) and NAA (0.5, 1.0 and 1.5 mg·L^{-1}) as factors. The callus induction rate and regeneration coefficient were calculated after 40 days.

Simultaneous medium for callus induction, adventitious bud occurrence and proliferation. According to the results of a single factor and complete combination experiment, different concentrations of 6-BA (2.0, 3.0 and 4.0 mg·L^{-1}), NAA (0.5, 1.0 and 1.5 mg·L^{-1}), and KT (0.5, 1.0 and 1.5 mg·L^{-1}) were added to the MS medium for the design of the L_9 (3^4) orthogonal test. The orthogonal design was shown in Table 1. After 40 days, the adventitious bud occurrence coefficient (statistical standards: the height of adventitious bud not less than 0.5 cm) and regeneration coefficient were calculated.

Improved medium of browning. After 2–3 subcultures in the synchronous medium for callus proliferation, adventitious bud occurrence and proliferation, serious browning appeared in the callus. Therefore, 1 g·L^{-1} activated carbon (AC) was added to the optimal synchronous medium. After 40 days, adventitious buds occurrence coefficient and regeneration coefficient were calculated.

Medium for rooting. As the basic medium for rooting ½ MS with or without 1 g·L^{-1} AC was used, in which different concentrations of NAA (0.0, 0.5, 1.0, and 1.5 mg·L^{-1}) were added, respectively. After 45 days, the rooting rate and the average number of adventitious roots per plant were counted.

Inoculation method and culture conditions. To establish an aseptic system, 250 mL cans of one explant per bottle were used. Subsequently, 500 mL culture flasks were used for each subsequent stage. Among them, for single factor experiment, each treatment was inoculated with 5 vials and each vial contained 10 explants. Each treatment for complete combination and orthogonal experiment was inoculated with 10 bottles, each bottle of 12 explants. For rooting culture, each treatment was inoculated with 20 vials of 7 explants per vial. At the proliferation stage, callus was cut from the surrounding of the explant, divided into 0.7–1.0 × 0.7–1.0 cm (with 2–3 bud points) in size, and inserted into the medium. In the rooting stage, the main buds with a height of 2–3 cm and a strong basal stem were selected from cluster buds, and the callus were cleared and then inserted into the rooting medium. In addition to the establishment of a sterile system, the

Table 1. L_9 (3^4) orthogonal design for callus induction, adventitious buds occurrence and proliferation

Level	Factors (mg·L^{-1})		
	A (6-BA)	B (NAA)	C (KT)
1	2.0	0.5	0.5
2	3.0	1.0	1.0
3	4.0	1.5	1.5

Note: 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin

above treatment was repeated 3 times, if the explant was dead or contaminated, timely supplement.

The incubation temperature was controlled at 25 ±2 °C. The illumination intensity was maintained at 2,000–2,500 lx, and the illumination time was 12 $h \cdot d^{-1}$.

Acclimation and transplanting

Rooting in 1/2MS medium with 1.0 $mg \cdot L^{-1}$ NAA for 40 days, the height of *in vitro* plant was about 8–10 cm, with a well-developed root system; the culture bottle was moved from the culture room to the greenhouse and placed for 2–3 days. Next, the cap was removed and placed under natural light for 2–3 days, with a small amount of tap water added for another 1–2 days (prevented growth of bacteria in the medium). Afterwards, *in vitro* plants were gently removed with tweezers, followed by clean water to remove the culture medium attached to the roots, and soaked in 0.1% carbendazim solution (w/v) for 10–30 min. Finally, they were transplanted into plastic pots (10 cm in diameter) containing mixed soil in a ratio of peat soil : coconut bran : perlite : yellow mud (4 : 1 : 1 : 2). After 60 days, the survival rate was counted (temperature 20–25 °C and humidity about 70%).

For field transplanting, plantlets were planted in a health farm in Maguan County, Wenshan City, Yunnan Province, China (103°95'E, 22°89'N, 418–460 m a.s.l.). The long-term annual average temperature and total annual precipitation are 21.9 °C and 1,500 mm, respectively.

Statistical statistics

The collected data were processed and analyzed using SPSS 26.0 (IBM Corp., Armonk, NY) and Excel (MC Corp., Redmond, WA). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test to assess significant differences among treatments, with a significance threshold of $P < 0.05$. Adobe Photoshop (2021) and Origin (2021) software were conducted for image processing.). The calculation formula was as follows:

Contamination rate (%) = the number of contaminated explants/total number of explants × 100%

Survival rate of explant (%) = the number of surviving explants/total number of explants × 100%

Callus induction rate (%) = the number of explants with the effective callus/total number of initial inoculations × 100%

Adventitious bud occurrence coefficient = the number of effective adventitious buds/the number of calluses

Regeneration coefficient = the number of effective subculture/total number of initial inoculations

Rooting rate = the number of single shoots with adventitious roots/total number of single shoots inoculated × 100%

The survival rate = the number of surviving plants/total number of transplanting plants × 100%

RESULTS

Establishment of an aseptic system

A certain difference in the contamination and survival rate of explants treated with different disinfection time was revealed (Tab. 2). When sterilized for 8 min, the sterilization was insufficient, but the contamination rate was as high as 73.33% and only a few explants could survive. When disinfection time reached 18 min, the contamination rate was lowered to 6.67%, but the survival rate was only 26.67% due to a strong toxicity of $HgCl_2$ and the intolerance of explants. With the prolongation of sterilization time, the contamination rate of explants decreased, and the survival rate also decreased correspondingly. In each treatment, a certain number of explants were uncontaminated without the sign of growth, and then gradually died. No significant difference in explant survival between 12 min and 14 min disinfection ($P > 0.05$) but a significant difference in contamination rate was found ($P < 0.05$). Overall, optimal disinfection time was 14 min (33.3% contamination rate and 46.67% survival rate).

After 40 days of culture, signs of budding on the leaf base were found (Fig. 1A). After 60 days, green protuberance appeared (Fig. 1B). After 80 days, 3–4 buds appeared in each explant (Fig. 1C and D).

Initial culture and single factor experiments

Obtained individual buds from an aseptic system were cut off and transferred to the initial medium. Af-

Table 2. Effects of different sterilization time on contamination and survival of *A. comosus* explants

Sterilization time (min)	Number of inoculated	Contamination rate (%)	Survival rate (%)
8	15	73.33 ±4.221 a	6.67 ±5.637 d
10	15	60.00 ±5.250 ab	20.00 ±11.547 c
12	15	46.67 ±6.667 b	40.00 ±9.667 a
14	15	33.33 ±5.267 c	46.67 ±6.667 a
16	15	13.33 ±6.258 d	33.33 ±13.333 b
18	15	6.67 ±5.756 e	26.67 ±5.543 c

Note: Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

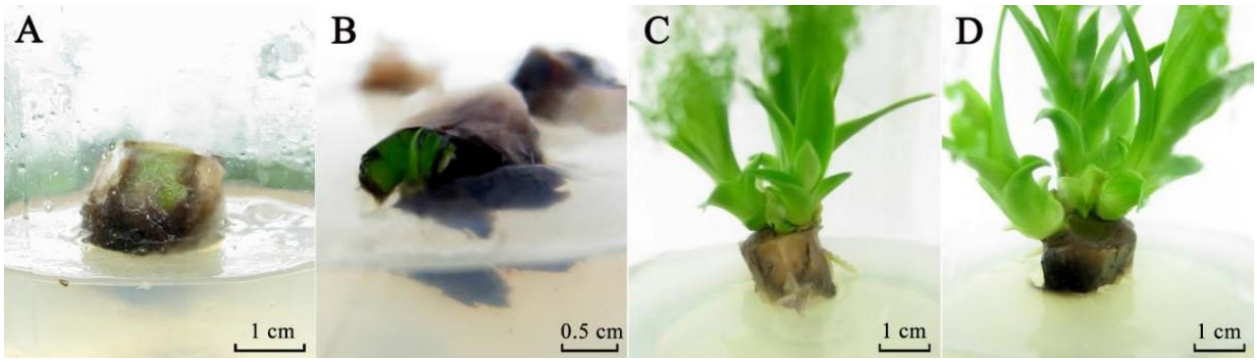

Fig. 1. Establishment of aseptic system

(A) The growth condition after 40 days of culture; (B) The growth condition after 60 days of culture; (C and D) The growth condition after 80 days of culture

ter 40 days, 2–3 lateral buds appeared at the base of the stem (Fig. 2A), or a small amount of callus was produced at the base, accompanied by the appearance of weak adventitious buds (Fig. 2B), with 2.25 regeneration coefficient (Tab. 3). Although ZT, added to initial culture, showed significant effect in promoting buds regeneration, due to its high cost, this study decided to replace it with some more affordable PGRs, such as 6-BA, KT, 2, 4-D, and NAA.

Growth of the bud of pineapple was observed in all treatments (Fig. 2 and Tab. 3). Among them, 6-BA treatment showed a significant effect on responses to callus induction as inducing a large amount of compact callus. However, almost no adventitious buds occurred, especially at the concentration of 2.0–4.0 mg·L^{-1}, with 68.5% callus induction rate (Fig. 2C). Of all KT treatments, a certain number of calluses and weak adventitious buds appeared at the base of main buds, and no significant difference among different levels was found (Fig. 2D). No callus was observed in 2, 4-D treatment with all levels. Compared with cytokinin treatment, buds in this treatment grew faster and clumped roots appeared at the base (Fig. 2E). NAA treatment significantly promoted the growth of buds, while a large number of adventitious roots occurred. *In vitro* shoots were well-grown with the strong roots, especially in 1.0–1.5 mg·L^{-1} NAA treatment (Fig. 2F).

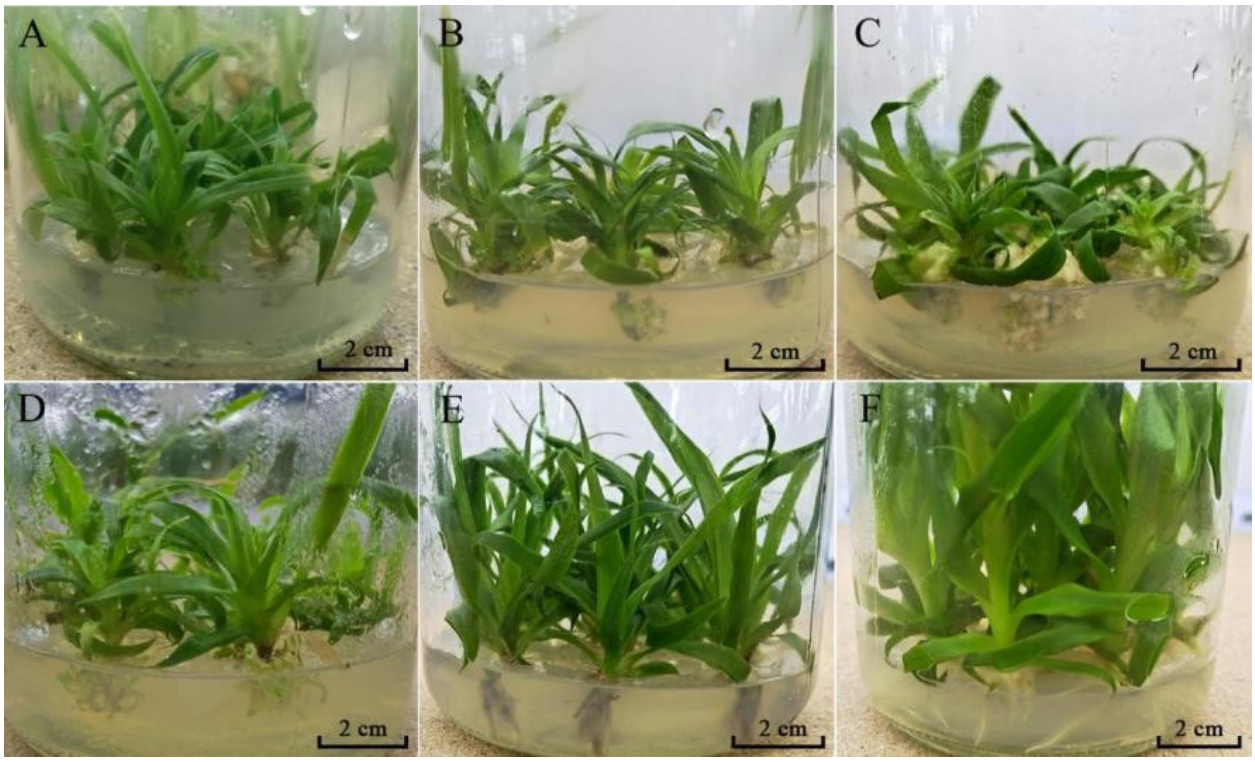

Fig. 2. Initial culture and single factor experiments

(A) After 40 days of initial culture, 2–3 new buds appeared in the base of a part of explants; (B) After 40 days of initial culture, a small amount of callus appeared in the base of a part of explants, accompanied by adventitious bud differentiation; (C) A large number of white calluses with compact in texture appeared in MS medium with 3.0 mg·L^{-1} 6-BA; (D) In MS medium with 1.0 mg·L^{-1} KT, a small amount of callus appeared in the base, with adventitious bud differentiation; (E) The growth potential in MS medium with 0.05 mg·L^{-1} 2, 4-D was perferable to that in cytokinin treatment, and the adventitious roots were clumped at the base; (F) In MS medium with 1.0 mg·L^{-1} NAA, the explant grew vigorously, adventitious roots developed, without the appearance of callus and lateral bud

Callus induction

As can be seen from Table 4, a significant difference between PGR treatment and blank control (CK) was revealed. Growth in CK treatment was not completely stopped; it is estimated to be that used explants were all from the single factor experiment, which had accumulated certain exogenous PGRs. In addition, the occurrence rate of callus was 100% in all treatments except CK, but a significant difference in the regeneration coefficient was observed. When the concentration of NAA remained constant, regeneration coefficient showed an upward trend within 2.0–3.0 mg·L^{-1} 6-BA. When the concentration of 6-BA was 3.0 mg·L^{-1}, this treatment had the highest regeneration coefficient, which was significantly different from other treatments ($P < 0.05$). The regeneration coefficient began to decrease when the ratio was above 3.0 mg·L^{-1}. When 6-BA concentration remained unchanged, the comparison of C_4 (3.0 mg·L^{-1} 6-BA+ 0.5 mg·L^{-1} NAA), C_5 (3.0 mg·L^{-1} 6-BA+ 1.0 mg·L^{-1} NAA) and C_6 (3.0 mg·L^{-1} 6-BA+ 1.5 mg·L^{-1} NAA) treatments showed that the regeneration coefficient of callus was related to the concentration of NAA, and a significant difference among treatments was revealed ($P < 0.05$). Thus, the MS medium containing 3.0 mg·L^{-1} 6-BA and 0.5 mg·L^{-1} NAA (C4) was optimal medium for callus induction.

In the C_4 treatment, after 10 days of culture, the base began to swell and white callus appeared (Fig. 3A). After 20 days, every base of verticillate leaf expanded to form white callus (Fig. 3B). After 30 days, callus grew and proliferated rapidly (Fig. 3C). After 40 days, most

Table 3. Effects of different types and concentrations of PGRs on buds, callus, and roots of *A. comosus* explants

PGRs (mg·L^{-1})		Regeneration coefficient	Callus induction rate (%)	Rooting rate (%)
Initial medium (MS with 1.5 mg·L^{-1} ZT)		2.25 ±0.09 a	13.33 ±3.33 e	0.00
6-BA	1.0	1.50 ±0.12 b	43.33 ±3.33 c	0.00
	2.0	1.03 ±0.03 cd	60.00 ±5.77 ab	0.00
	3.0	1.17 ±0.03 d	68.50 ±3.33 a	0.00
	4.0	1.03 ±0.03 d	53.33 ±3.33 b	0.00
KT	0.5	1.03 ±0.03 d	33.33 ±3.33 d	0.00
	1.0	1.10 ±0.06 d	36.67 ±3.33 cd	0.00
	2.0	1.07 ±0.07 d	30.00 ±3.33 d	0.00
2, 4-D	0.01	1.17 ±0.09 cd	0.00	33.33 ±3.33 d
	0.05	1.03 ±0.03 d	0.00	63.33 ±3.33 c
	0.1	1.30 ±0.01 c	0.00	40.00 ±5.78 d
NAA	0.5	1.03 ±0.03 d	0.00	70.00 ±5.78 bc
	1.0	1.00 ±0.00 d	0.00	100.00 ±0.00 a
	1.5	1.00 ±0.00 d	0.00	100.00 ±0.00 a
	2.0	1.00 ±0.00 d	0.00	76.67 ±3.33 b

Note: 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

Table 4. Effects of 6-BA and NAA combination on callus induction

Medium	PGRs (mg·L^{-1})		Regeneration coefficient
	6-BA	NAA	
CK	0	0	1.33 ±0.125 f
C_1	2.0	0.5	3.99 ±0.178 cd
C_2	2.0	1.0	3.51 ±0.245 cd
C_3	2.0	1.5	2.68 ±0.138 e
C_4	3.0	0.5	5.33 ±0.179 a
C_5	3.0	1.0	4.63 ±0.098 b
C_6	3.0	1.5	3.80 ±0.253 cd
C_7	4.0	0.5	4.18 ±0.283 bc
C_8	4.0	1.0	3.66 ±0.211 cd
C_9	4.0	1.5	2.86 ±0.169 e

Note: PGRs – plant growth regulators; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; CK – blank control. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ± SE

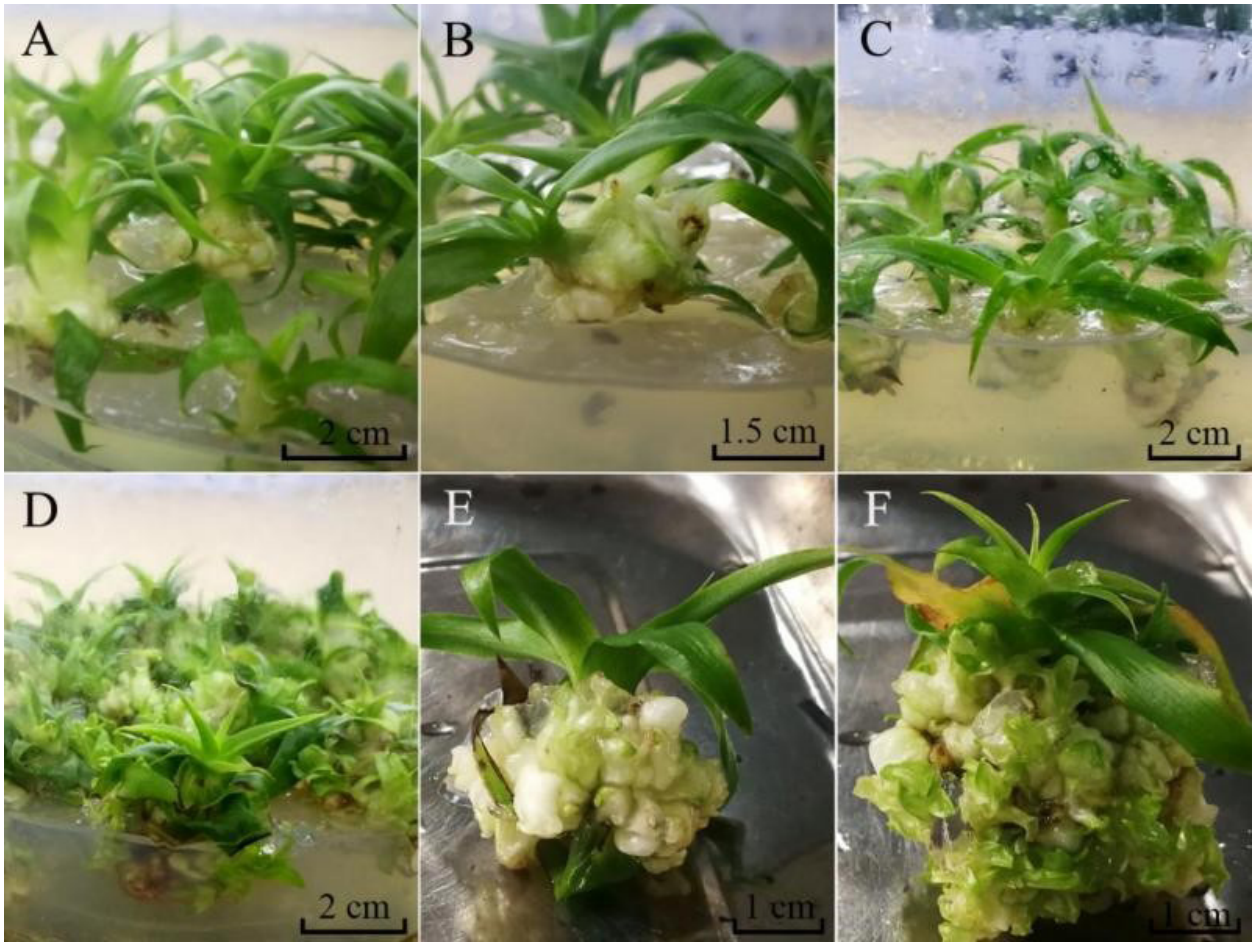

Fig. 3. Callus induction

(A) White enlargement callus appeared at the base of the bud after 10 days of culture; (B) After 20 days of culture, callus also appeared at the base of the leaf; (C) After 30 days of culture, the base of each bud expanded and new callus appeared, with a fast regeneration rate; (D) After 40 days of culture, the whole surface of the medium was basically covered by callus; (E and F) Layered callus with green protuberance

of the buds were covered by callus except for the top leaf (Fig. 3D). At this time, callus appeared at the axils of each leaf, presenting a unique stratified phenomenon (Fig. 3E, F).

Simultaneous culture for callus proliferation, adventitious bud differentiation and proliferation

In order to further explore an effective method of callus redifferentiation into adventitious cluster buds and improve proliferation efficiency, another PGR (KT) was introduced for orthogonal experiment according to the results of single factor and complete combination experiment, and the results were shown in Table 5.

The range (R) analysis in Table 5 shows that $R_{KT} > R_{6\text{-BA}} > R_{blank} > R_{NAA}$, indicating that the response of KT and 6-BA were effective in adventitious buds occurrence coefficient, among which KT had the best response, while NAA had no significant response. According to the results of variance analysis (Tab. 6), KT showed a significant response on adventitious bud occurrence coefficient ($P < 0.05$), while 6-BA and NAA had no significant response ($P > 0.05$). Duncan test (Tab. 7) for the three levels of KT shows that level 2 (1.0 mg·L^{-1}) had the greatest response on the adventitious bud occurrence coefficient, which was significantly different from level 1 (0.5 mg·L^{-1}) and level 3 (1.5 mg·L^{-1}). According to the levels of each factor

Table 5. Results of L_9 (3^4) orthogonal experiment on callus proliferation, adventitious bud differentiation and proliferation

Medium		PGRs (mg·L^{-1})				ABOC	RC
		A (6-BA)	B (NAA)	C (KT)	D (Error)		
1		2.0	0.5	0.5	(1)	6.11 ±0.015	5.98 ±0.103
2		2.0	1.0	1.0	(2)	10.02 ±0.105	7.97 ±0.053
3		2.0	1.5	1.5	(3)	8.12 ±0.372	6.96 ±0.061
4		3.0	0.5	1.0	(3)	11.76 ±0.040	8.72 ±0.046
5		3.0	1.0	1.5	(1)	9.86 ±0.050	8.02 ±0.031
6		3.0	1.5	0.5	(2)	7.64 ±0.101	7.21 ±0.100
7		4.0	0.5	1.5	(2)	8.99 ±0.208	8.93 ±0.131
8		4.0	1.0	0.5	(3)	9.63 ±0.036	8.68 ±0.174
9		4.0	1.5	1.0	(1)	12.53 ±0.026	10.44 ±0.079
ABOC	*K*	8.083	8.953	7.793	9.500	–	–
	K	9.753	9.837	11.437	8.883	–	–
	K	10.383	9.430	8.990	9.837	–	–
	R	2.300	0.884	3.644	0.954	–	–
RC	*K*	6.970	7.877	7.290	8.147	–	–
	K	7.983	8.223	9.043	8.037	–	–
	K	9.350	8.203	7.970	8.120	–	–
	R	2.380	0.346	1.753	0.110	–	–

Note: PGRs – plant growth regulators; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – Kinetin; ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; *K* – average; *R* – range

in the L_9 (3^4) orthogonal design table (Tab. 1) and the average value in L_9 (3^4) orthogonal experiment results (Tab. 5), the optimal combination of PGRs for adventitious bud occurrence was $A_3B_2C_2$ (level 3 of 6-BA, level 2 of NAA, and level 2 of KT), namely 4.0 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} NAA + 1.0 mg·L^{-1} KT.

The descending order of regeneration coefficient was shown in Table 5: $R_{6\text{-BA}} > R_{KT} > R_{NAA} > R_{blank}$, and the *R* values of 3 PGRs were all higher than the blank column, indicating that three PGRs were effective in proliferation effect, of which 6-BA had the best effect, followed by KT and NAA. According to the results of variance analysis (Tab. 6), 6-BA had a significant effect on the regeneration coefficient ($P < 0.05$), while KT and NAA had no significant effect ($P > 0.05$). Duncan test (Tab. 7) was carried out on three levels of 6-BA. For the regeneration coefficient, level 3 (4.0 mg·L^{-1}) had the best influence, which was significantly different from that of level 1 (2.0 mg·L^{-1}) and level 2 (3.0 mg·L^{-1}). By means of average value analysis, the optimal combination of PGRs for regeneration coefficient was also $A_3B_2C_2$, namely 4.0 mg·L^{-1} 6-BA + 1.0 mg·L^{-1} NAA + 1.0 mg·L^{-1} KT.

The optimal combination was obtained by repeating the orthogonal experiment for 3 times. After 10 days of culture, adventitious buds began to appear on the surface of callus (Fig. 4A and B). After 20 days of culture, with the continuous differentiation of adventitious buds, the callus proliferated rapidly (Fig. 4C and D). After 30 days, early adventitious buds grew vigorously, and new adventitious buds were constantly differentiated (Fig. 4E and F). After 40 days,

Table 6. Variance analysis of occurrence coefficient of adventitious bud and regeneration coefficient

Factor	Sources	Type III sum of square	DF	Mean square	F value	Significance
ABOC	A (6-BA)	8.476	2	4.238	1.093	$P > 0.05$
	B (NAA)	1.173	2	0.586	0.115	$P > 0.05$
	C (KT)	20.692	2	10.346	5.617	$P < 0.05$
	D (Error)	1.402	2	0.701		
RC	A (6-BA)	8.559	2	4.280	5.202	$P < 0.05$
	B (NAA)	0.227	2	0.114	0.051	$P > 0.05$
	C (KT)	4.689	2	2.344	1.597	$P > 0.05$
	D (Error)	0.020	2	0.010		

Note: ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; DF – degree of freedom; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin

Table 7. Three levels of KT and 6-BA Duncan test

Factor	PGRs	Level	Mean
ABOC	KT	2 (1.0 mg·L^{-1})	11.437 a
		3 (1.5 mg·L^{-1})	8.990 ab
		1 (0.5 mg·L^{-1})	7.793 b
RC	6-BA	3 (4.0 mg·L^{-1})	9.350 a
		2 (3.0 mg·L^{-1})	7.983 ab
		1 (2.0 mg·L^{-1})	6.970 b

Note: ABOC – adventitious bud occurrence coefficient; RC – regeneration coefficient; 6-BA – 6-benzylaminopurine; KT – kinetin; PGRs – plant growth regulators. The different letters following the mean indicate a significant difference ($P < 0.05$) between the levels of KT or BA corresponding to the mean

the surface of callus was covered by adventitious buds (Fig. 4G and H). At this point, adventitious buds occurrence coefficient was 12.80, and regeneration coefficient of subculture was 10.38.

After 2–3 generations of culture in the synchronous medium for callus proliferation, adventitious bud occurrence and proliferation, callus showed serious browning (Fig. 5A). At this time, adding 1.0 mg·L^{-1} AC could completely eliminate callus browning. After 10 days, callus began to differentiate adventitious buds (Fig. 5B). After 20 days, adventitious buds grew rapidly, and callus also proliferated rapidly (Fig. 5C and D). After 30 days, callus was further proliferated, and adventitious buds on the callus grew vigorously (Fig. 5E and F). After 40 days, the whole medium was covered by adventitious buds, and 3–5 robust main buds appeared on each callus, which was conducive to the next step of rooting culture (Fig. 5G and H). At this time, the occurrence coefficient and the proliferation coefficient of adventitious bud didn't significantly reduce. Browning did not appear in the subsequent subculture proliferation.

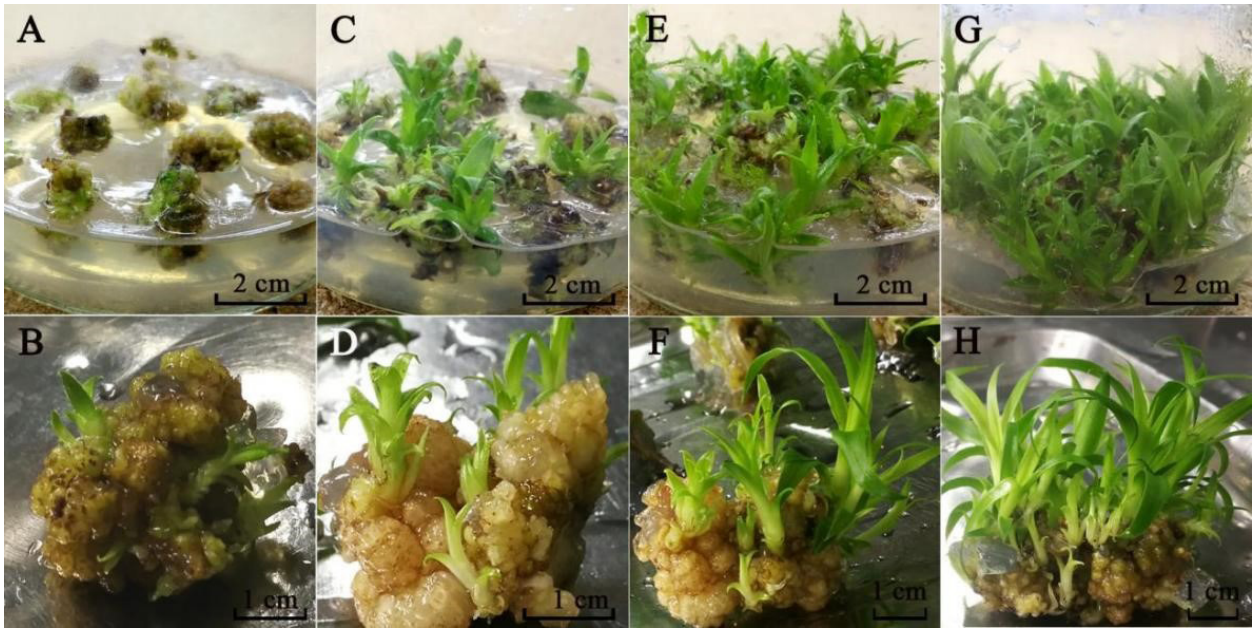

Fig. 4. Synchronous culture for callus proliferation, adventitious bud differentiation and proliferation

(A and B) After 10 days of culture, green adventitious buds appeared on the surface of callus; (C and D) After 20 days, callus proliferated rapidly with the increase of the number of adventitious buds; (E and F) After 30 days, early adventitious buds had grown into plantlets, and new adventitious buds were constantly differentiated from callus; (G and H) After 40 days, the whole surface of callus was covered by adventitious buds

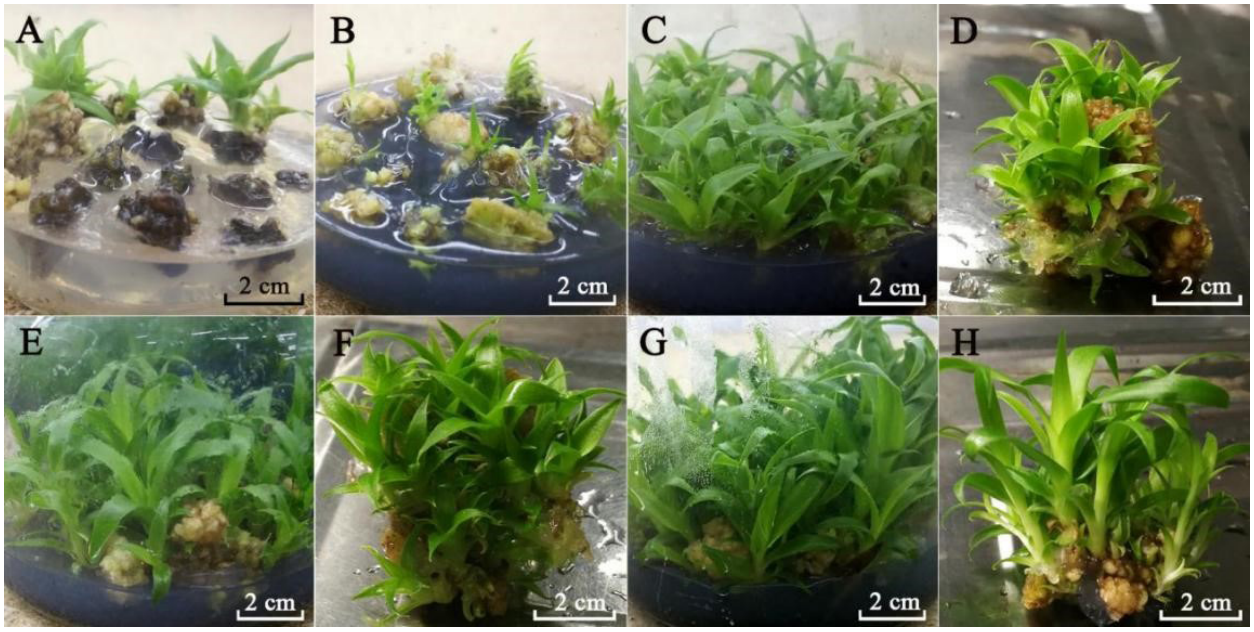

Fig. 5. Improvement of callus browning and proliferation culture

(A) Browning callus that lost the ability to proliferate and redifferentiation; (B) To alleviate the problem of callus browning, 1.0 mg·L^{-1} AC was added to the culture medium; After 10 days of culture, adventitious buds began to differentiate on the surface of callus; (C and D) After 20 days, callus also proliferated rapidly to adapt to the rapid growth of adventitious buds; (E and F) After 30 days, adventitious buds grew vigorously and stimulated callus to proliferate further; (G and H) After 40 days, adventitious buds occupied the whole surface of the medium, with 3–5 healthy main buds per cluster buds

Rooting culture

The rooting rate of all treatments including CK was 100%. It can be seen from Table 8 that the treatment without AC was more suitable for induction and growth of adventitious roots than the treatment with AC. The number of adventitious roots per plant in NAA treatment was significantly higher than that in CK ($P < 0.05$), which proved that auxin NAA had a significant effect on root induction. The number and growth of adventitious roots increased in a positive ratio with the increase of the concentration in 0.5–1.0 mg·L^{-1} NAA. However, the number of adventitious roots decreased and the growth trends of *in vitro* shoot became weaker as over 1.0 mg·L^{-1} NAA. In other words, a higher concentration of auxin not only inhibited the occurrence of adventitious roots, but also was not

Table 8. Rooting culture

Medium	NAA (mg·L^{-1})	The number of adventitious roots	Explant characteristics
Without AC			
CK-1	0.0	2.36 ±0.39 d	pale green leaves; few roots with slender; *in vitro* shoot was weak with a slow growth rate; a small amount of callus
R_1	0.5	4.67 ±0.52 b	plenty of bright green leaves; stout and long roots; a good growth trend of *in vitro* shoot
R_2	1.0	7.35 ±0.44 a	dark green leaves; plenty of stout roots; a good growth trend of *in vitro* shoot
R_3	1.5	4.34 ±0.47 b	green leaves; stout and short roots with a poor toughness; a slow growth trend
With AC (1.0 g·L^{-1})			
CK-2	0.0	1.31 ±0.51 e	small number of green leaves; a few slender roots; slender *in vitro* shoot; a poor growth trend; a small amount of callus
R_4	0.5	3.35 ±0.37 cd	green leaves; a few slender roots
R_5	1.0	3.69 ±0.61 c	leaflet; a slow growth trend; underdeveloped root; a poor growth trend
R_6	1.5	2.68 ±0.46 d	green leaflet; slow to take root; a poor growth trend

Note: AC – Activated carbon; NAA – α-naphthaleneacetic acid. Different letters in the same column indicate significant differences at $P < 0.05$. Data are mean ±SE

conducive to the growth of *in vitro* shoot. Therefore, the optimal induction medium for adventitious roots of pineapple was 1/2MS medium with 1.0 mg·L^{-1} NAA. In this medium, after 15 days of culture, white root tips appeared around the base of *in vitro* shoot (Fig. 6A and B). After 25 days, leaves were extended, new leaves appeared continuously, plant height was obvious, and the growth of adventitious root was visible with the elongation of adventitious root (Fig. 6C and D). After 35 days, the basal stem of *in vitro* shoots became thick, adventitious roots became thick and elongated with obvious root hairs (Fig. 6E and F). After 45 days, *in vitro* shoots grew robust, with developed and robust adventitious roots at the bottom, which was suitable for acclimation and transplanting (Fig. 6G and H).

Acclimation, transplanting and variation

After acclimation, the survival rate of *in vitro* plant was 100% (Fig. 7A and B). After 4 months, the plant grew healthily, new leaves were constantly appeared, old leaves were wider and longer, and the height of the plant was about 15–20 cm, which met the requirements of field planting (Fig. 7C and D). After 8 months of field planting, inflorescence expanded with the appearance of the fruit (Fig. 7E). There was no significant difference in the size and taste of the fruit between the tissue culture seedings and the mother plants after acclimation and transplanting after 12 months (Fig. 7F).

Notably, the variation of about 0.01% leaves of *in vitro* shoots was observed during proliferation or rooting (Fig. 8A and B). Due to a small number, the variation was not tracked in this study and was discarded immediately after discovery. In addition, no similar phenomenon was observed in the process of acclimation and transplanting.

DISCUSSION

In this study, direct plantlet regeneration from leaf base had poor problems, including a low reproduction coefficient and a weak plant. In addition, to obtain re-

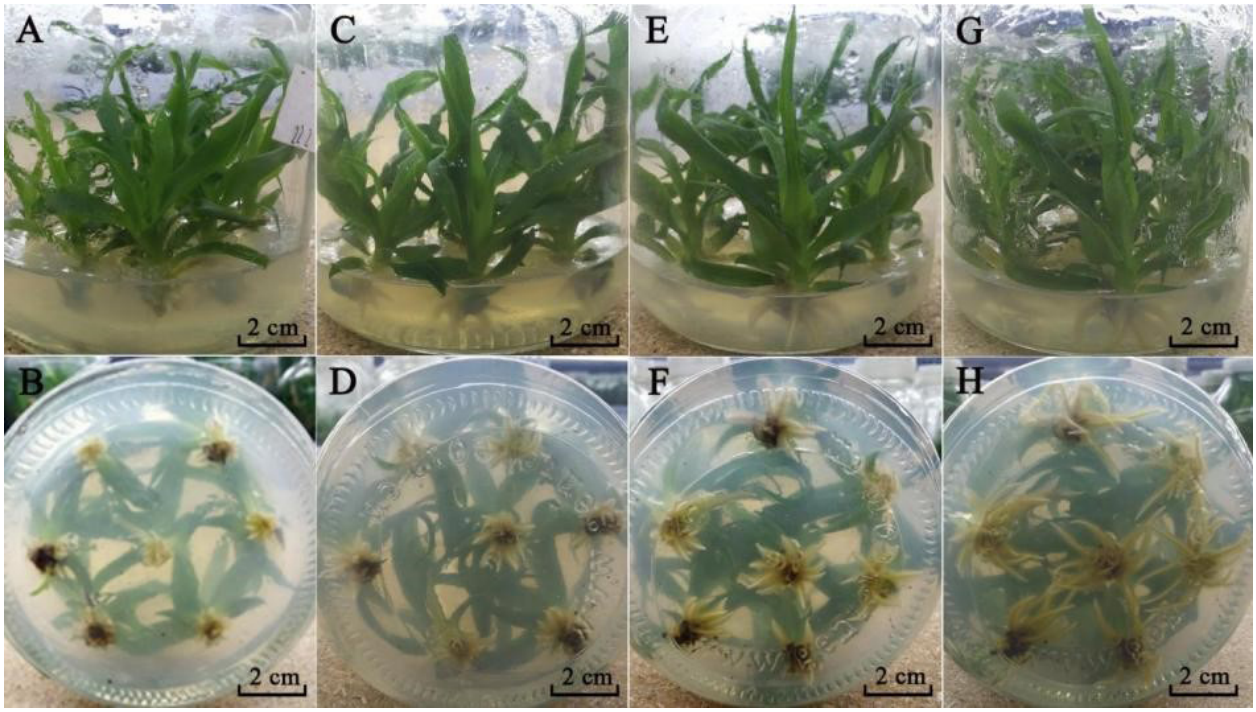

Fig. 6. Rooting culture

(A and B) After culture for 15 days, *in vitro* shoot grew obviously, and adventitious roots grew in a wheel shape at the bottom of the bottle; (C and D) After 25 days, *in vitro* shoots grew further and adventitious roots also grew rapidly; (E and F) After 35 days, *in vitro* shoots were thickened and adventitious roots had developed root hairs; (G and H) After 45 days, *in vitro* shoots with strong and thick roots were the best acclimation material

Fig. 7. Acclimation, transplanting and variation

(A) *In vitro* plant acclimation for 15 days; (B) After 60 days of acclimation, the roots of *in vitro* plant were developed; (C) Plant after 4 months; (D) After 5 months of acclimation, the leaves were red because of low temperature; (E) After 8 months of transplanting, fruit began to appear; (F) After 12 months of transplanting, the fruit was ripe

generated plants on a large scale, it could only be done on the premise of sufficient number of explants, and it was quite difficult to operate. Thus, this method was only suitable for the initial culture stage. For indirect organogenesis, it was found that the appearance of callus was closely related to the rupture of leaf base, that was, the expansion of callus from the bottom to the top resulted in the rupture of leaf base of each whorl, which resulted in the emergence of new callus. After proliferation, each leaf could be wrapped up, presenting a unique stratified phenomenon. Because of this phenomenon, the number of calluses was significantly increased, which also proved that the leaf base of pineapple has a strong potential for dedifferentiation [Firoozabady and Moy 2004]. The leaf base located near the axillary meristem may contain meristem regions or newly developed meristem, which can rapidly divide cells and construct organ morphology during culture. Interestingly, in the existing reports of *in vitro* rapid propagation of pineapple [Soneji et al. 2002a, 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Cacaï et al. 2023, Mendonça et al. 2017, Lakho et al. 2023, Torres Ruiz et al. 2023], callus stage was rarely mentioned, and most of them

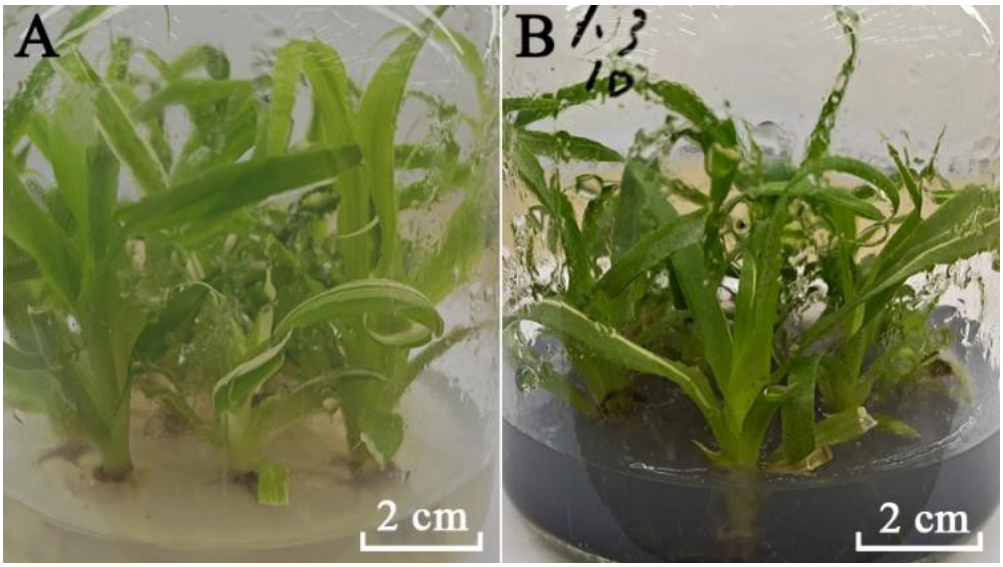

Fig. 8. Phenotypic variation; (A and B) Albino striped variegated foliage

were replaced by bud aggregate. However, in an early stage of this study, callus had obvious and strong proliferative effect, and only after the adventitious cluster buds appeared did the bud aggregates with numerous buds formed on the carrier of callus. Another difference was that adventitious buds produced from callus in this study grew well. In the period of proliferation, these adventitious buds showed a complete morphology of regenerated plants. However, according to the descriptions or pictures of other researchers, adventitious buds were weak and basically had no regenerated plant morphology, which requires repeated rejuvenation culture [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Reinhardt et al. 2018]. Also, the callus in this study proliferated quickly, and high propagation coefficient could be obtained through a subculture in a short period of time, which reduced the high labour cost and production cost caused by repeated rejuvenation. In addition, it has been reported that in *in vitro* rapid propagation of pineapple, genotype had a strong influence on the regeneration method [Da Silva et al. 2016]. Whether this view is applicable to the object of this study still needs to be verified by similar studies on other varieties or variants of pineapple.

It was reported that there was no ideal method for *in vitro* propagation of specific plant species at present, and micropropagation needed to be adjusted and improved for each specific explant in order to achieve higher efficiency [Reinhardt et al. 2018]. The methods of callus induction, adventitious bud occurrence and proliferation, and plant regeneration for a variant pineapple were established in this study. Current reports in different varieties of pineapple, adventitious buds occurrence and proliferation could be achieved by using high concentration of 6-BA alone (5.0–8.0 mg·L^{-1}) or in combination with auxin such as NAA, IAA or IBA [Soneji et al. 2002b, Firoozabady and Gutterson 2003, Sripaoraya et al. 2003, Be and Debergh 2006, Zuraida et al. 2011, Ibrahim et al. 2013, Usman et al. 2013, Nelson et al. 2015, Scherer et al. 2015, Da Silva et al. 2016, Mendonça et al. 2017, Cacaï et al. 2023, Lakho et al. 2023, Torres Ruiz et al. 2023]. Different from the results of previous studies, only part of the explants used cytokinin in this study produced callus, and the culture effect was far from expected. Auxin alone showed a significant effect on the rooting rejuvenation of the explant. When 6-BA and NAA were combined, the callus induction rate significantly increased, and the best treatment could reach 100%. However, adventitious bud differentiation was less, even if differentiation, there were signs of growth inhibition. Thus, KT was selected for orthogonal experiment. Not only callus proliferation was greatly increased, but also differentiation coefficient of adventitious buds was remarkably increased. These results indicated that KT could not only promote cell division, but also induce bud differentiation and

development. It also proved that the synergistic effect produced by multiple factors was more conducive to the proliferation culture of the study object.

Serious browning appeared after further propagation culture. Surprisingly, although pineapple tissue culture has been widely reported, only Soneji et al. [2002a] have mentioned this phenomenon and no improvement measures have been taken. In addition, according to related reports, the addition of AC in tissue culture could not only prevent oxidation, but also remove most inhibitory and toxic substances, even substances released during culture, thus reducing browning [Lizeth and María de Lourdes 2018]. Therefore, in this study, AC was added at this stage, and browning was basically eliminated, which indicated that AC had a positive role in reducing the browning of this variety of pineapple.

In the culture after the elimination of browning, the proliferation of callus and adventitious buds was complementary to each other. The proliferation of callus clearly occurred after the appearance of adventitious buds. In a certain culture space, the more adventitious buds, the faster the proliferation of callus. Generally, plant endogenous auxin is produced at the tip of the stem, while cytokinin is produced at the tip of the root. In this study, the concentration of cytokinin was much higher than that of auxin. It was estimated that after the occurrence of adventitious buds, the growth hormone synthesized by bud tip might be transported down rapidly and combine with the exogenous cytokinin accumulated in callus, thereby promoting callus proliferation. At this time, the process of callus induction could be removed in this study, and callus proliferation and adventitious bud occurrence were carried out simultaneously in 6-BA, NAA and KT combinations, with a proliferation period of only 40–45 days. This kind of simultaneous culture was not only effective but also has a high regeneration coefficient, which achieved the goal of efficient and rapid propagation.

CONCLUSIONS

This study successfully established methods for callus induction, adventitious bud formation, and plant regeneration in the new line of *A. comosus* selected from a spontaneous mutant of cultivar Yellow Mauritius. Callus formation was closely linked to the rupture of the leaf base, leading to increased callus generation. The stratified phenomenon of leaf wrapping further enhanced callus proliferation, demonstrating the significant dedifferentiation potential of the pineapple leaf base. In this study, the addition of activated carbon (AC) effectively mitigated browning during propagation, highlighting its beneficial role in this process. Ultimately, the study achieved high callus proliferation and a high propagation coefficient through rapid subculture, thereby reducing labour and production costs associated with repeated rejuvenation.

AUTHORS' CONTRIBUTIONS

The author confirms their contributions to the manuscript as follows: Writing-Original Draft Preparation, Validation, Visualization: CL; Formal analysis, Funding acquisition, Project administration: FX; Writing-review & editing, Conceptualization, Methodology, Supervision, Writing-review & editing: HH. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

All authors declare no conflict of interest.

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