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RESEARCH PAPER

Developmental responses of perennial ryegrass, red fescue, and Kentucky bluegrass to *In vitro* chitosan treatments

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Abstract

Effects of chitosan oligomers and polymer on *in vitro* development of perennial ryegrass (*Lolium perenne* L.), red fescue (*Festuca rubra* L.), and Kentucky bluegrass (*Poa pratensis* L.) were studied to elucidate a possible differentiation between the effects of chitosan depending on its chemical structure. The seed germination rate was enhanced after the oligomer treatments. The oligomer mixture triggered leaf elongation better than the polymer. However, the highest number of leaves was found from *L. perenne* in the polymer's presence at 10 mg·L^{-1} in the medium. The maximum leaf length was reached in *L. perenne* after oligomeric chitosan treatment at 5 mg·L^{-1}. The plant's rhizogenic response was enhanced in *P. pratensis* but decreased in *L. perenne* and *F. rubra* after 2.5 mg·L^{-1} oligomeric chitosan treatment. However, the root elongation was restricted in *F. rubra* and *P. pratensis* after chitosan treatments. Conversely, chitosan treatments augmented root elongation in *L. perenne*. This study suggested that chitosan might be preferred to ensure better turf coverage in these grass species. However, constant- or over-treatment with chitosan could reduce root growth and increase the plant's leaf elongation that might contribute to nutritional deficiency and increased mowing costs, respectively.

Introduction

Perennial ryegrass (*Lolium perenne* L.), red fescue (*Festuca rubra* L.), and Kentucky bluegrass (*Poa pratensis* L.) are cosmopolitan, cool season, and perennial grass species with dense turf production ability (Ayan et al., 2020). These species are individually cultivated for a number of uses, such as forage production, lawn production for ornamental purposes, and recreational events. *L. perenne* and *F. rubra* are also used for soil stabilization. *P. pratensis* is not preferred for soil stabilization since it has a shallow root system (Wennerberg, 2004). However, the mixture of *P. pratensis* and *L. perenne* is advantageous in establishing a more disease-resistant turfgrass with better color and year-round growth (Wilen et al., 2009). These grass species can also grow on most soil types since they have a wide range of adaptability to most soils (St. John et al., 2012; Acemi, 2021). Several cultivars of the *P. pratensis* and *L. perenne* have been shown to tolerate salt, while some cultivars of *F. rubra* have been reported to perform under drought and heat stresses (Marcum & Pessarakli, 2010; Wang et al., 2017; Bushman et al., 2020). The properties mentioned above of these species make them desirable grass species in forage and lawn production. Therefore, more scientific studies should be focused on such multipurpose species. The conventional propagation method is seed sowing for these species, although creeping species *F. rubra* and *P. pratensis* can be produced by rhizome cutting, whereas *L. perenne,* a non-creeping plant, has a fibrous root system. Therefore, cultivation practices for the improvement of desired traits in such plants also include the enhancement of seed germination rate as well as morphometric parameters of leaf and root development.

The effects of synthetic fertilizers and growth regulators are being tested to regulate growth and improve seed yield, visual quality, and traffic tolerance in many turfgrass species (McMahon & Hunter, 2012; Trethewey et al., 2016). However, natural substances with growth-promoting activities are continuously being discovered as alternatives to synthetic chemicals used on agricultural, horticultural, and ornamental plants. In this sense, the deacetylation of chitin biopolymer extracted primarily from shells of crustaceans and cell walls of fungi led researchers to produce chitosan (Tan et al., 2020), which is considered to be one of the alternatives to synthetic growth-promoters (Acemi et al., 2018). Chitosan is a linear aminopolysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine (GlcNAc). Chitosan exhibits various effects on plants, such as enhancing seed germination, stimulating plant growth, inducing biological responses to abiotic and biotic stresses, and extending the shelf life of vegetables, ornamentals, and fruits (Romanazzi et al., 2016; Hidangmayum et al., 2019; Acemi, 2020a). Chitosan's structure may vary depending on its degree of polymerization (DP), which reflects the number of monomeric units in the polymer, and the degree of acetylation (DA), representing the molar fraction of GlcNAc in the polymer. These differences in chitosan's chemical structure have been shown to be decisive on the variation of its effects on horticultural plants, suggesting a structure-function relationship in chitosan's chemical structure and its function in plants (Acemi, 2020b).

The current study aimed to answer two research questions. The first of these questions is whether there is a possible differentiation among commercially available grass species' responses to chitosan treatments. The second question is whether there is a possible differentiation between the effects of chitosan samples with different DPs on commonly cultivated grass species. Therefore, the former question places the grass species into the focus of the research while the latter focuses on the effects of chitosan's structure on its function on grass species' development. By taking advantage of the plant tissue culture technique, we aimed to answer these research questions through a controlled culture environment that eliminates the other factors contributing to the plants' development, thereby focusing only on the elucidation of chitosan's effects on *L. perenne*, *F. rubra*, and *P. pratensis*. Based on the preceding reports referred above, it is hypothesized that oligomeric and polymeric chitosan samples should also lead to different effects on the *in vitro* development of *L. perenne*, *F. rubra*, and *P. pratensis* while enhancing the seed germination and promoting the growth of the species. Also, the determination of the *in vitro* effects of well-characterized chitosans on widespread and commonly used grass species would illustrate the possible usability of chitosan as a natural alternative to synthetic growth-promoters in turfgrass cultivation and forage production.

Materials and Methods

Chitosans' source and characterization

The chitosan samples were previously produced, characterized, and provided by the Institute of Plant Biology and Biotechnology, University of Münster, Münster, Germany. The origin of the chitosan samples was shrimp shell wastes. The polymer that had the DP of 70 was previously analyzed using HP-SEC-RID MALLS following Schatz et al. (2003), while MALDI-TOF-MS was used to characterize the oligomers which had DPs ranging from 2 to 15 (Haebel et al., 2007). The DA of the samples (10%) was previously determined through 1H-NMR (Vårum et al., 1991).

Cultivars, and seeds' source, disinfection, and transplantation

The seeds of *Lolium perenne* cv. Esquire, *Festuca rubra* cv. Maxima1, and *Poa pratensis* cv. Evora were provided by the local dealer (Sekoya Tohumculuk Ziraat San. & Tic. A.Ş, Turkey) of DLF Seeds Ltd., Denmark. The seeds were kept at dark, dry, and cool place until use. A hundred seeds of each species were placed into bags (4 × 4 cm) prepared from filter paper. The seeds were then kept in 1% (w/v) sodium hypochlorite (NaOCl) solution for 8 min for disinfection. The excess NaOCl on the seeds was removed by rinsing them into sterile water several times. The bags were then opened using a sterile blade, and the seeds were transplanted onto the medium using sterile forceps. All the treatments were carried out in a laminar airflow cabinet.

Media preparation and culture conditions

The culture vessels (Magenta GA-7) were filled with 40 ml of Murashige and Skoog's medium (Murashige & Skoog, 1962) supplemented with a mixture of chitosan oligomers with DPs ranging from 2 to 15, or polymer with a DP of 70 at 2.5, 5, or 10 $mg \cdot L^{-1}$ concentrations. Sucrose at 30 $g\ L^{-1}$ concentration was used as a carbon source, and the medium was solidified using 7 $g \cdot L^{-1}$ agar. One N NaOH or HCl was used to balance the pH of the medium at 5.7. The medium was sterilized through autoclaving at 121°C under a pressure of 118 kPa for 20 min. The chitosan samples were filter-sterilized and added to the medium after autoclaving. The surface-sterilized seeds were placed horizontally onto the culture medium, and the culture vessels were then incubated in a plant growth chamber. The photosynthetic photon flux density striking to the cultures was 60 $\mu mol\ m^{-2} \cdot s^{-1}$ with a 16-h photoperiod, and the temperature was 23 ± 1°C. The incubation period consisted of 30 d starting after the transplantation of the seeds onto the medium.

Data collection and visualization, and statistical analysis

The measurements of the morphological parameters were done at the end of the incubation period. Each treatment was tested on 20 seeds in each repeat, and the experiments were done with five replications. Data were represented as mean ± standard deviation (SD). Duncan's multiple range test at a $P < 0.05$ significance level was used to compare the means after the one-way analysis of variance (ANOVA) was conducted. Statistical comparisons were made through IBM SPSS Statistics 22 software. The developmental data were standardized and shown through heatmaps for each species to visualize the degrees of the species' responses given to the treatments comparatively. The morphological differences and similarities caused by treatments in each species were analyzed through hierarchical cluster analysis (HCA) based on the Euclidean distance and complete-linkage clustering method. The clustering heatmap was created through ClustVis (Metsalu & Vilo, 2015).

Results

Effect of chitosan on seed germination rate

The germination of the seeds occurred within the first week of the incubation period. The chitosan variants did not affect the seed germination rate in *L. perenne*. The control medium gave 90 ± 3.54% mean seed germination in *L. perenne*, while the lowest mean seed germination rates (88 ± 6.71% and 88 ± 5.70%) were calculated from the medium with chitosan polymers at 5 and 10 mg·L^{-1} concentrations, respectively. However, the medium with chitosan polymer at 2.5 mg·L^{-1} gave the maximum mean seed germination rate (95 ± 3.54%) in the same species (Figure 1).

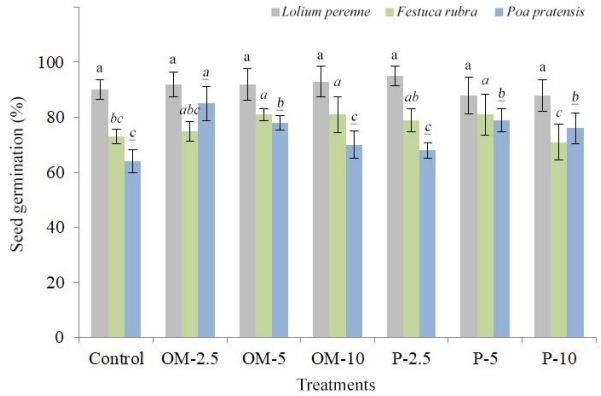

Figure 1. Comparison of the effects of chitosan treatments on *in vitro* germination of *Lolium perenne*, *Festuca rubra*, and *Poa pratensis* seeds. Data represent mean ± SD. The bars with the same-style superscript letters are not significantly different by Duncan's multiple range test ($P < 0.05$).

The mean seed germination rate from the control medium was found 73 ± 2.74% in *F. rubra*. The oligomer mixture at 5 and 10 mg·L^{-1} and polymer at 5 mg·L^{-1} increased the mean seed germination rate to 81 ± 2.24%, 81 ± 6.52%, and 81 ± 7.42%, respectively. However, the medium with chitosan polymer at 10 mg·L^{-1} gave the lowest (71 ± 6.52%) mean seed germination rate (Figure 1).

In *P. pratensis*, the control medium gave the lowest seed germination rate (64 ± 4.18%), while the medium with chitosan oligomers at 2.5 mg·L^{-1} concentration increased the mean germination rate up to 85 ± 6.12%. The mean seed germination rates from the medium with chitosan polymer at 5 and 10 mg·L^{-1} were statistically the same as that of the medium with chitosan oligomers at 5 mg·L^{-1} (Figure 1).

Effect of chitosan on leaf development

Chitosan treatments greatly influenced leaf formation. The mean leaf numbers per plant from the control medium were found 1.97 ± 0.05, 1.63 ± 0.17, and 1.17 ± 0.08, respectively, for *L. perenne*, *F. rubra*, and *P. pratensis*. The mean leaf numbers calculated from the control groups were also the minimum values for the plants. All the chitosan treatments tested significantly increased leaf production in all species. The maximum mean leaf number in *L. perenne* (2.76 ± 0.13) was found from the medium with chitosan polymer at 10 mg·L^{-1}, while an increasing trend was observed in leaf numbers with the elevated chitosan concentrations. In *F. rubra*, all the chitosan treatments gave statistically the same results, while the highest mean leaf number (2.17 ± 0.09) was calculated from the medium with chitosan polymer at 5 mg·L^{-1}. A similar trend in the same parameter was also observed in *P. pratensis*. The chitosan treatments significantly increased the mean leaf numbers in *P. pratensis*, and the maximum value (2.26 ± 0.06) for the parameter was reached from the medium with chitosan oligomers at 2.5 mg·L^{-1} (Figure 2a).

Leaf elongation was also triggered after chitosan treatments. The chitosan oligomers induced longer leaves than polymer treatments. The control medium gave the mean leaf lengths 7.12 ± 0.52 cm, 6.50 ± 0.74 cm, and 2.54 ± 0.29 cm per leaf in *L. perenne*, *F. rubra*, and *P. pratensis*, respectively. The control groups also gave the lowest mean leaf lengths. The most elongated leaves in *L. perenne* (11.51 ± 0.26 cm) were found from the medium supplemented with chitosan oligomers at 5 mg·L^{-1}. In *F. rubra*, all the chitosan treatments induced close leaf lengths. The highest mean leaf length per leaf (9.76 ± 0.25 cm) in *F. rubra* was found from the medium with chitosan oligomers at 10 mg·L^{-1}. *P. pratensis* showed a similar response with *F. rubra* to the chitosan treatments. The most elongate leaves in *P. pratensis* (5.20 ± 0.30 cm) were measured from the medium with 5 mg chitosan oligomers at 5 mg·L^{-1} (Figure 2b).

Effect of chitosan on root development

Chitosan treatments affected the root formation differently in all the grass species employed in the study. However, the most significant changes were found in *P. pratensis*. The maximum number of roots per plant (3.96

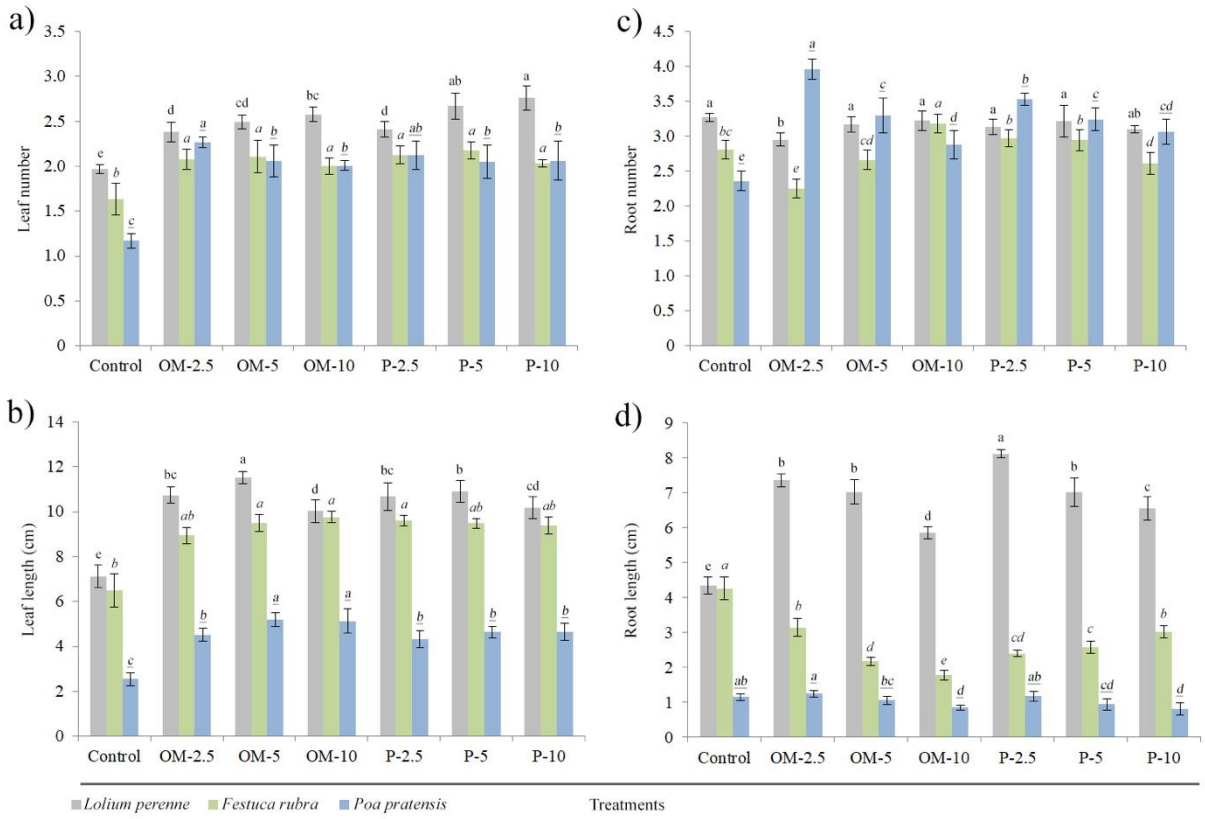

Figure 2. Comparison of the effects of chitosan treatments on *in vitro* **a**) leaf production, **b**) leaf elongation, **c**) rhizogenesis, and **d**) root elongation in *Lolium perenne*, *Festuca rubra*, and *Poa pratensis*. Data represent mean ± SD. The bars with the same-style superscript letters are not significantly different by Duncan's multiple range test ($P < 0.05$).

± 0.15) was produced in the medium with chitosan oligomers at 2.5 mg·L^{-1} concentration, while the control group gave the lowest number of roots (2.36 ± 0.14) in *P. pratensis*. The control medium in *L. perenne* and *F. rubra* gave 3.27 ± 0.06 and 2.81 ± 0.13 roots per plant, respectively. The chitosan treatments reduced the root production in *L. perenne*, and the minimum mean number of roots (2.95 ± 0.10) was found from the medium with chitosan oligomers at 2.5 mg·L^{-1}. In *F. rubra*, the detractive effects of chitosan on root production become more evident in the presence of oligomers at decreasing and the polymer at increasing concentrations. However, the polymer at 5 and 10 mg·L^{-1} in the medium gave the same results statistically. The minimum mean number of roots (2.25 ± 0.25) was calculated from the medium with chitosan oligomers at 2.5 mg·L^{-1} (Figure 2c).

In contrast to chitosan's growth-promoting effects on leaf and root formation in *P. pratensis*, root elongation reduced after chitosan treatments. The root elongation-inhibitory effect of chitosan oligomers was more evident than the polymer in *F. rubra*. However, chitosan treatments increased root lengths in *L. perenne*. The minimum mean root length (4.34 ± 0.24 cm) for *L. perenne* was recorded from the control group, whereas the most extended mean root length (8.12 ± 0.13 cm) was found from the medium supplemented with chitosan polymer at 2.5 mg·L^{-1}. In *F. rubra*, the control medium gave the highest (4.26 ± 0.33 cm) mean root length. However, increasing concentrations of chitosan oligomers and elevated concentrations of the polymer reduced the mean root length. The medium with chitosan oligomers at 10 mg·L^{-1} gave the shortest roots (1.78 ± 0.13 cm). The highest concentration of chitosan oligomers led to a reduction of root elongation in *P. pratensis*. The minimum mean root length was found 0.80 ± 0.17 cm per plant after chitosan polymer treatment at 10 mg·L$^{-1,}$ which was statistically the same as that of the oligomers at 10 mg·L^{-1} (Figure 2d).

Comparison of the development patterns through normalized data

In all cluster analyses, control groups were found in a separate cluster than the chitosan treatments. The polymer treatments at moderate and high concentrations were placed next to each other, while other treatments were more closely grouped in the HCA analysis for *L. perenne* (Figure 3a). At its lowest level, the oligomer treatment was found in the same cluster with the polymer treatment at the highest concentration, whereas the other treatments were found in a closer relationship in the HCA analysis for *F. rubra* (Figure 3b). In the HCA analysis for *P. pratensis*, the oligomer and the polymer treatments at moderate and high concentrations were closely grouped, whereas the lowest concentrations of both treatments were found in neighboring clusters (Figure 3c).

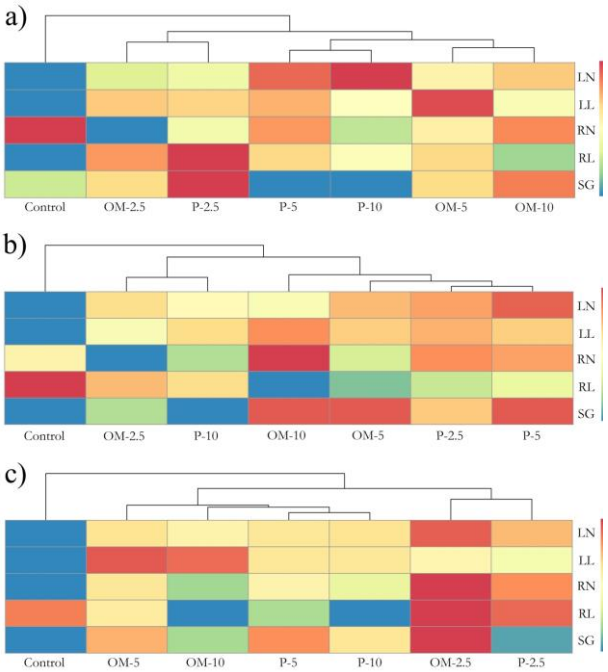

Figure 3. Hierarchical clustering heatmap-based comparison of the normalized developmental data from **a**) *Lolium perenne*, **b**) *Festuca rubra*, and **c**) *Poa pratensis*. Leaf number (LN), Leaf length (LL), Root number (RN), Root length (RL), Germination rate (SG), Chitosan polymer (P), Chitosan oligomers (OM). The treatments are represented as "chitosan variant – concentration ($mg \cdot L^{-1}$)".

Discussion

In turfgrass and forage management, selection and application of suitable fertilizers, growth regulators, and other types of growth-promoting chemicals according to the plants' needs cover a significant place in the cultivation practices' success. Turfgrass species are led to invade and cover the fields mainly used for sports activities at the beginning of their cultivation. The turf coverage, which is dependent on the foliar growth performance of turf species, is then enhanced through chemical fertilizers or growth promoters. However, many synthetic growth regulators such as Trinexapac Ethyl, Paclobutrazol, or Ethephon are applied primarily to suppress seedhead production (type I growth regulators) or to inhibit cell elongation (type II growth regulators) for better mowing practices and visual quality of turfgrass following the successful establishment of turf cover (Głąb et al., 2020). On the other hand, more plant biomass but lesser control of plant growth than turf grass cultivation is needed for forage production that mostly depends on vegetative parts' growth (Capstaff & Miller, 2018). Therefore, enhancement of seed germination rate and plant growth by using a biodegradable, eco-friendly, and natural growth-promoter such as chitosan would be beneficial for producers, sustainability, and nature.

To date, various growth regulators and other chemicals have been studied on turfgrass species in order to reveal the mechanisms controlling their growth and alleviating the effects of several environmental stress factors (Ma et al., 2018; Głąb et al., 2020). However, a limited number of reports regarding the effects of chitosan application on seed germination in turfgrass species are found in the literature. In one of the reports, Kim (2014) treated *P. pratensis* with uncharacterized chitosan and reported early germination. In the present study, chitosan treatments' success in enhancing seed germination rate was demonstrated in a concentration-dependent manner. However, chitosan oligomers better enhanced the germination rates in *P. pratensis* and *F. rubra* than the polymer, whereas seeds of *L. perenne* showed a limited positive response to chitosan treatments. This finding can be explained by reducing the medium's osmotic potential through polymeric chitosan treatment, whose hydrophilic nature is proportional to the polymer's chain length (Acemi, 2020a). Therefore, lesser osmotic potential reduction in the culture medium might be expected when oligomers are used at low concentrations. Seeds need to imbibe a higher amount of water for germination, and lower osmotic potentials of the culture medium would limit the water uptake of the seeds. The reduction of germination rate in *P. pratensis*, *F. rubra*, *L. perenne*, and other turfgrass species such as *Schedonorus arundinaceus*, *Festuca brevipila*, and *F. rubra* ssp. *fallax* after decrement of osmotic potential has been demonstrated on a prediction-based model (Goatley et al., 2017). In the study, the authors reported that the seeds of *P. pratensis* are the most susceptible to the osmotic potential changes, whereas the seeds of *L. perenne* are the most tolerant of such changes among other turfgrass species. The authors also noted that *L. perenne* seeds had the highest germination rate, which is in line with our findings. The better ability of chitosan oligomers than polymers in enhancing seed germination rate in *P. pratensis* and *F. rubra* (Figure 1) might also be explained by their higher potential to stimulate the production of reactive oxygen species (ROS) in the seeds. Because ROS could play a role in regulating seed germination by oxidizing the proteins that trigger germination (El-Maarouf-Bouteau et al., 2013) and weakening the endosperm during seed swelling (Müller et al., 2009). In a recent report, the dormancy release was associated with increasing the sunflower seeds' internal H_2O_2 level (Vigliocco et al., 2019).

Leaf production and growth are considered among the parameters that determine the visual quality and cover ability of turfgrass, and the forage yield is also strictly dependent on the same parameters. The present analysis showed that chitosan successfully supported the above-listed growth parameters in the grass species tested (Figure 2a&b). Chitosan treatments have been shown to induce the synthesis of several plant growth regulators, such as benzyladenine (BA) and indole 3-acetic-acid (IAA), which involve regulating the meristematic cell division and organogenesis (Jogaiah et al., 2020). The same researchers also found that chitosan, when applied at a specific concentration,

induced callose and lignin deposition in the cucumber plant (Jogaiah et al., 2020). The promotive effects of chitosan in leaf production and elongation might be attributed to these effects possibly found also in the grass species tested in this study.

On the other hand, synthesis of callose, which is a cell wall polymer synthesized during cytokinesis and practically involved in the cell division process (Thiele et al., 2009) beside regulation of plasmodesmata and stomata closure (Nedukha, 2015), was found to be triggered in *Phaseolus vulgaris* after chitosan treatments (Franco & Iriti, 2007). Here, it should be noted that callose is degraded to form cellulose to support cell wall growth after cytokinesis (Nedukha, 2015). Also, increased cell wall lignification through the stimulation of lignin biosynthesis after chitosan treatment was reported by Acemi and Türker-Kaya (2020). However, Mondal et al. (2012) stated that chitosan's growth-promotive effects might be due to increased enzyme activities in nitrogen (N) metabolism and the increased N transportation. Therefore, the better leaf elongation performance of *L. perenne*, *F. rubra*, and *P. pratensis* treated with chitosan oligomers in this study might be explained by oligomers' better ability to trigger the synthesis of plant growth regulators and other biomolecules involved in cell division. Also, chitosan oligomers' superiority in the same parameter might be attributed to its better ability to enhance N metabolism and transportation than the polymer. Furthermore, increased leaf production after chitosan treatment was also reported in *Lactuca sativa* (Xu & Mou, 2018), *Curcuma longa* (Anusuya & Sathiyabama, 2016), and *Ipomoea purpurea* (Acemi et al., 2018), while enhanced chlorophyll content and visual quality in *P. pratensis* treated with chitosan was reported by Chang and Yoon (2011).

Grass root growth that extends deep into the soil is one of the most significant factors helping prolong grass life and reduce fertilizer use. The current study's findings indicated that chitosan use induced a reliably more robust rhizogenic response in *P. pratensis* than *F. rubra* and *L. perenne* (Figure 2c). However, root elongation was reduced in *P. pratensis* when both of the chitosan variants were used above 5 $mg{\cdot}L^{-1}$ (Figure 2d). In *F. rubra*, chitosan treatments reduced the root elongation regardless of its DP. However, *L. perenne* was the only grass species with longer roots after chitosan treatments (Figure 2d). In a recent report that the authors showed the alteration in auxin homeostasis and the accumulation of IAA after chitosan polymer (DP 70, DA 15%) treatments between 0.1 and 1 $mg{\cdot}mL^{-1}$, arrested root elongation in the apical root meristem of *Arabidopsis* after chitosan treatments was reported (Lopez-Moya et al., 2017). The authors attributed their findings to the reduced expression of the WUSCHEL-RELATED HOMEOBOX 5 (*WOX5*) gene, which controls the stem cells' activity in the quiescent center of the root tissue where the cell division is regulated. Therefore, increased root production, however, reduced root elongation in *F. rubra* and *P. pratensis,* might be explained by the possible accumulation of IAA and downregulation of *WOX5*, respectively. However, this discussion should be proven with further studies, and the physiological mechanism behind chitosan's success in triggering root elongation in *L. perenne* should be investigated. In the root tissues of another monocotyledonous plant, *Serapias vomeracea*, chitosan polymer treatment was reported to decrease water-associated cellulose content, while oligomer treatment led to an increase in the same parameter (Acemi & Türker-Kaya, 2020). In other reports conducted with uncharacterized chitosan, increased leaf number, chlorophyll content, and fresh and dry weight were reported in *P. pratensis* (Yoon & Kim, 2007) and *Agrostis palustris* (Yoon et al., 2006) treated with 500× diluted chitosan solution. In those studies, the species were cultured in soil, where their roots were not continuously in contact with chitosan due to soil drainage. However, in tissue culture, the culture medium is more stable and has no drainage like soil, making the roots exposed to the test treatments continuously. This condition might be the reason behind that the researchers in both reports found longer roots in the plants treated with chitosan than control, which is partly in contrast with the current study.

In light of the outcomes derived from the analyses, the possible use of chitosan should be taken into consideration to enhance foliar growth, which would be a favorable trait in turf and forage production. However, it should be noted that root growth might decline in such cases, which would be a disadvantage for turfs encountering dense traffic and soil stabilization. Therefore, foliar application of chitosan should be tested in further field studies since foliar fertilization has a minimum impact on root growth (Liu et al., 2008). Also, molecular evaluation of the effects of chitosan on root development should be conducted to reveal the exact physiological mechanism behind its effects.

Conclusion

This study showed that the effects of chitosan treatment on turfgrass species could be altered in response to chitosan's chemical structure. Therefore, to ensure a high germination rate and improved leaf growth in *L. perenne*, *F. rubra*, and *P. pratensis* when establishing turf coverage on recreational fields, treatment with oligomers could be a better option than the polymer. However, excessive chitosan applications might reduce root development, which would lead to nutrient deficiency in the plants. After establishing turfgrass, continuous chitosan application would also increase mowing frequency due to leaf elongation and increase maintenance costs. For forage production, chitosan oligomers are suggested to be used more frequently in these species' cultivation since biomass production is more critical in such usage. It is necessary to reduce chemical fertilizers and growth regulators to

mitigate the harmful effects of cultivation on the environment. Therefore, characterized chitosan could be safely employed in the stages mentioned earlier of turfgrass and forage cultivation instead of synthetic growth regulators to minimize the harmful effects of excessive use of chemicals on nature. However, this suggestion should be tested in field conditions before large-scale application of chitosan.

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Author Contributions

Conceptualization: AA, Data curation: AA, DT, SY; Formal analysis: AA; Investigation: AA, DT, SY; Methodology: AA; Resources: FÖ; Visualization: AA; Writing - original draft: AA; Writing - review and editing: AA.

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BIOTECH STUDIES

RESEARCH PAPER

Characterization of intracellular β-galactosidase from *Bacillus subtilis* 4NK and *Bacillus paralicheniformis* 5NK isolated from a hot water spring and effects of various inhibitors on enzyme activity

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Abstract

In this study, the intracellular β-galactosidases of *Bacillus subtilis* 4NK and *Bacillus paralicheniformis* 5NK isolated from Bingöl Binkap hot spring was partially purified and characterized. As a result of purification, the yield of the enzyme for *B. subtilis* 4NK was 85.2% and the purification fold was 2.8. The yield for *B. paralicheniformis* 5NK was 76.8% and the purification fold was 2.0. The optimum temperature of the enzyme was determined as 45 °C for *B. subtilis* 4NK and 55 °C for *B. paralicheniformis* 5NK and the optimum pH was 6.0 for both. In addition, in the thermal stability experiments even at the end of 120 min both enzymes were stable at 50 °C. It was determined that the partially purified enzyme activity increased in the presence of iodoacetamide and phenylmethylsulfonylfluoride for *B. subtilis* 4NK, dithiothreitol, N-ethylenemaleimide and phenylmethylsulfonylfluoride for *B. paralicheniformis* 5NK. The metals were found to activate the enzyme at low concentrations of Co^{2+}, Cd^{2+} and Mn^{2+} for *B. subtilis* 4NK, Cu^{2+} and Cd^{2+} were found to inhibit the enzyme at high rates for *B. paralicheniformis* 5NK. K_m and V_{max} values for 4NK and 5NK, respectively; 23.80 mM, 1.978 µmol/min and 5.61 mM, 1.869 µmol/min.

Introduction

Thanks to the developing industrial enzyme technology and the development of fermentation methods, the production potential is increased in the production of enzymes of microbial origin, regardless of environmental conditions (Topal, 1998). Enzymes used in many fields in industry are generally obtained from microorganisms. Because microorganism-derived enzymes have higher catalytic activities compared to other sources, they do not form undesirable by-products (Kıran et al., 2006). Moreover, they can be produced through fermentation techniques in a cost-effective manner with less time and space requirement because of their high consistency, as well as easy process modification and optimization (Grung et al., 2013). In recent years, many studies have focused on thermophilic enzymes of microbial origin. Since thermostable enzymes purified from thermophilic ones maintain their stability even at temperatures exceeding the temperature at which the microorganism grows, these enzymes are more preferred (D'Auria et al., 1999). There are many advantages of thermophiles related to industrial use due to high growth rates accelerating processes of fermentation two to three times compared to those with mesophilic producers, less unwanted microbial contamination, as well as higher diffusion rate and mass turnover. Enzymes from thermophiles are well known to be more resistant to proteolysis and chemical denaturation. Thus, the process of enzyme "aging" is slowed down because of the stability of these molecules, which is preferred in commercial preparations allowing their storage at room temperature with a longer half-life (Kambourova, 2018). *B. paralicheniformis* is used in the biotechnology

industry to produce enzymes, antibiotics, biochemical and consumer products (Rey et al., 2004; Dunlap et al., 2015; Du et al., 2019).

β-galactosidase has been obtained from different microorganisms in the studies conducted so far. β-galactosidase (β-D-galactohydrolases, EC 3.2.1.23) enzymatically hydrolyses the β-glycosidic bond in lactose and produces glucose and galactose, which are sweeter than lactose and have higher solubility (Panesar, 2006). β-galactosidases are used for a variety of applications, including the production of dairy products, low-lactose milk and probiotics, biosynthesis of different transgalactose products, improvement of lactose tolerance, and various analytical approaches (Loveland et al., 1994; Neri et al., 2008) and they are also used in the production of other industrially important products such as ethanol and biosensors (Saqib et al., 2017).

As it is known, β-galactosidase is used in biotechnologically beneficial and important areas in the production of prebiotic food, in the processes of wastewater that occurs in the developing milk and dairy industry, production studies and post-production, and in the process of eliminating the problems caused by the milk products consumed by humans. In this study, isolation, partial purification and characterization of β-galactosidase from *B. subtilis* 4NK and *B. paralicheniformis* 5NK bacteria were aimed.

Materials and Methods

Biological Materials and Chemicals

In this study, *B. subtilis* 4NK and *B. paralicheniformis* 5NK were isolated and identified from Binkap hot springs in Ilısu district of Bingöl province (Aslan, 2018). Nutrient Broth (NB) and Agar were obtained from Merck Darmstatd, *o*-nitrophenyl-beta-D-galactoside (*o*NPG), dithiothreitol (DTT), N-ethylenemaleimide (NEM), iodoacetamide (IAA), phenylmethylsulfonylfluoride (PMSF) and *p*-chloromercuribenzoate (PCMB) were obtained from Sigma, ethylenediamintetraeceticacide (EDTA), $CaCl_2$, $CuCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$ and $CdCl_2$ were obtained from Merck Darmstatd, $MgCl_2$ was obtained from Kimetsan and $ZnCl_2$ from LACHEMA.

Cultivation of bacteria

B. subtilis 4NK and *B. paralicheniformis* 5NK bacteria were prepared using 1 mL inoculum each and incubated into 100 mL flasks with 25 mL NB, and incubated at 120 rpm at the optimum temperature for bacteria growth, using a shaker. The liquid culture was centrifuged at 10.000 rpm for 10 min and the pellet was sonicated by ultrasonication. The samples were centrifuged again under the same centrifuge conditions and the supernatant was used for β-galactosidase activity determination.

Determination of β-galactosidase activity

Enzyme activity was measured spectrophotometrically using *o*NPG determination of β-galactosidase activity. 60 mM *o*NPG was prepared by dissolving in 0.1 M Tris-HCl (pH 7.0) buffer in 10 mL and used as substrate. Enzyme source was obtained as a result of sonication of bacteria and added to the substrate. The mixture was incubated at 45 °C and 55 °C for 10 min. After the incubation, 1 M Na_2CO_3 was added in order to stop the reaction and measurement was made at 420 nm by spectrophotometer. Protein amount was determined using the Folin Reaction (Lowry et al., 1951). Unit enzyme is defined as the amount of enzyme that causes the release of *o*-nitrophenol in 1 min from 1 µM of *o*NPG substrate under certain conditions. The results are represented as the mean ± SD of at least 3 experiments.

Time and lactose dependent enzyme production

One mL of bacteria was inoculated separately into 250 mL of NB with 1% lactose and lactose-free NB (pH 7.0), and incubated for 48 hours and the bacteria reproduced at 6, 12, 24, 36, 48 hours. The culture was measured in the spectrophotometer at 600 nm. In addition, samples were taken from cultures centrifuged at 10.000 rpm for 10 min. After being centrifuged and sonicated, it was centrifuged again under the same centrifuge conditions and the supernatant was used for β-galactosidase activity determination and protein quantification.

Partial purification of β-galactosidase

Separate cultivation of bacteria was made on NB medium and incubated in shaker at 45 °C at 120 rpm for 24 hours. After incubation, samples were centrifuged at 10.000 rpm for 10 min. After the sonication process, the upper liquids were removed and used as crude extract. The ammonium sulphate was gradually added (70% for *B. subtilis* 4NK and 80% for *B. paralicheniformis* 5NK) into crude extracts and precipitation was performed in cold environment. The samples obtained after precipitation were centrifuged at 10.000 rpm for 25 min. The pellets were dissolved in 4 mL of 0.1 M Tris-HCl buffer (pH 7.0). Samples were dialyzed in 0.1 M Tris-HCl (pH 7.0) buffer at +4 °C overnight (approximately 18 hours) to remove ammonium sulphate from it using dialysis tubing. The final volume of each sample was calculated after dialysis. In order to concentrate the proteins in the solution more, the concentration process was carried out with the help of nitrogen gas using a stirred ultrafiltration system. The volume at the end of ultrafiltration was measured and transferred to a 1 mL sterile microcentrifuge tube and left at -20 °C. The specific activity, yield and purification fold of the enzyme were calculated by measuring the β-galactosidase activity and the protein amount of the samples was obtained from the crude extract and after dialysis.

Determination of optimum temperature and thermal stability

In order to determine the effect of temperature on the β-galactosidase activity, the partially purified enzyme activity was measured by incubating at different temperatures (25-65 °C). In order to determine the thermal stability of β-galactosidase activity, the enzyme activity was then measured in the temperature range of 45-60 °C for 10-120 min. For all these experiments, 50 u/mg of enzyme was used to investigate temperature effects and stability. The remaining activities after temperature exposures were calculated from the control (unheated enzyme) taking the activity as 100%.

Determination of optimum pH

To determine the effect of pH on the partially purified β-galactosidase activity, enzyme activities were determined using the prepared substrates in different buffers. The buffers were 0.1 M citric acid (pH 4.0-6.0), 0.1 M Tris-HCl (pH 7.0-8.0) and 0.1 M glycine-NaOH (pH 9.0- 11.0).

Effect of some chemicals and metals on enzyme activity

To determine the effect of some chemicals (DTT, PMSF, IAA, NEM, PCMB and EDTA) and metals (Mg^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+}) on the activity of enzymes, the partially purified enzyme was prepared separately with chemicals (NEM and PMSF dissolved in ethanol) and metals prepared in 0.1 M citric acid pH 6.0 buffer at different concentrations (1-10 mM for chemicals, 1-20 mM for metals and EDTA). It was left to pre-incubate for 15 min. Then, the enzyme activity was measured under optimum conditions determined for each enzyme by adding substrate. Samples with no added chemicals or metals were used as controls. Divalent metals were used in chloride form. For all these experiments, 50 u/mg of enzyme was used to investigate inhibition effects of chemicals tested. The remaining activities after chemical exposures were calculated from the control (untreated enzyme with any chemicals) taking the activity as 100 %.

Enzyme kinetics

In order to determine the substrate specificity, the partially purified enzyme was incubated at the optimum pH and temperature using *o*NPG (at concentrations of 1-10 mM) as substrate and the β-galactosidase enzyme activity was measured. *Km* and *Vmax* values of *o*NPG were calculated using Linewear-Burk equation.

Results and Discussion

In recent years, studies have focused on the identification of bacteria isolated from extreme conditions such as hot springs and their important products (Poli et al., 2006). Organisms have developed cellular cell membranes and cellular metabolites (such as enzymes and proteins) and some functional adaptations in order to live and reproduce in extreme conditions (Haki & Rakshit, 2003; Reed et al., 2013). The most important feature of thermophilic bacteria isolated from hot water springs is that they have enzymes that are resistant to extreme conditions, which makes them biotechnologically important (Demirjian et al., 2001). In this study, it was determined that *B. subtilis* 4NK and *B. paralicheniformis* 5NK bacteria isolated and identified from Binkap hot water spring in Bingöl province have a biotechnologically important β-galactosidase production potential. In addition, this study is the first regarding β-galactosidase studies on *B. paralicheniformis*.

Time and lactose dependent enzyme production

Time-course experiments between 6-48 hours were carried out to investigate lactose effects on enzyme production, in comparison to lactose-free controls. The highest enzyme activity of *B. subtilis* 4NK was obtained at 24 hours in lactose and lactose-free medium, and at 36 hours (22.62 U/mg) in lactose medium (Figure 1).

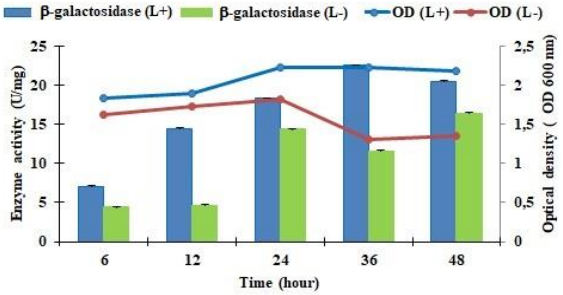

Figure 1. Time and lactose dependent enzyme production for *B. subtilis* 4NK (using NB with 1% lactose and lactose-free NB (pH 7.0), incubated at 40 °C for 48 h).

For *B. paralicheniformis* 5NK, the highest bacterial growth was obtained at 12th hour in lactose and lactose-free medium within 6-48 hours and the best enzyme activity was obtained at 48th hour (91.17 U/mg) in lactose-free medium (Figure 2).

For both bacteria, the enzyme production increased due to the increased lactose concentrations, indicating that their synthesis is constitutive (essential enzyme). Hirata et al. (1985), in their study, showed that with *B. subtilis* the synthesis of the enzyme was increased by inducing it in the presence of lactose and that the enzyme was synthesized constitutively.

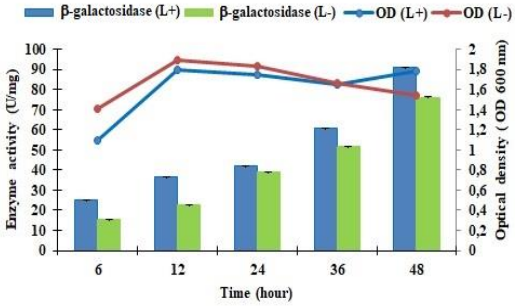

Figure 2. Time and lactose dependent enzyme production for *B. paralicheniformis* 5NK (using NB with 1% lactose and lactose-free NB (pH 7.0), incubated at 45 °C for 48 h).

Partial purification of β-galactosidase

The enzyme was partially purified by ammonium sulphate precipitation and dialysis. As a result of this purification, the purification fold was 2.8 and the yield was 85% for *B. subtilis* 4NK, the purification fold was 2 and the yield was 76.8% for *B. paralicheniformis* 5NK (Table 1). Literature data reveal some strategies for purifying β-galactosidases from microorganisms. Isobe et al. (2013a, b) purified β-galactosidase from *Teratosphaeria acidotherma* reaching a purification factor and yield of 375 and 2.9%, respectively. Martarello et al. (2019) purified β-galactosidase from fungi reaching a purification factor of 8.665 and a yield of 17.33%.

Optimum temperature and thermal stability

As seen in Figure 3, the effect of the temperature between 25-65 °C on the enzyme activity was investigated and β-galactosidase was active between 35-55 °C and the optimum temperature was 45 °C for *B. subtilis* 4NK. It was determined that the enzyme was active between 50-60 °C and the optimum temperature was 55 °C for *B. paralicheniformis* 5NK. With this feature, the enzyme shows that it can be used in processes such as the hydrolysis of lactose and whey processes, especially in the dairy industry, and it will also provide an advantage in reducing the risk of microbial contamination in processes that require high temperatures in these industries. In previous studies related to β-galactosidase obtained from various microorganisms, maximum β-galactosidase activity was obtained from *Streptococcus thermophilus* (Somkuti & Steinberg, 1979), *Lactobacillus kefiranofaciens* K-12

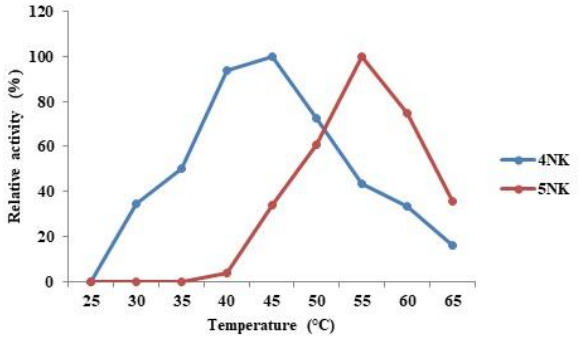

Figure 3. Effect of temperature on β-galactosidase (Optimum temperature was determined under standard assay conditions using *o*NPG at temperatures ranging from 25 to 65 °C).

(Itoh et al., 1993), *B. subtilis* (Torres and Lee, 1995), *B. circulans* (Vetere & Paoletti, 1998), *Bacillus* sp. MTCC 3088 (Chakraborti et al., 2000), *B. coagulans* RCS3 (Batra et al., 2002), *A. acidocaldarius* (Di Laura et al., 2008), *Anoxybacillus* sp. KP1 (Matpan Bekler et al., 2017) and *Anoxybacillus* sp. FMB1 (Yalaz et al., 2019) at 55 °C, 50 °C, 50 °C, 44 °C, 60 °C, 65 °C, 60 °C, respectively.

In Figure 4, the thermal stability of β-galactosidase enzyme activity was examined at 40-50 °C in the range of 10-120 min for *B. subtilis* 4NK. The enzyme was stable at 45 °C for 30 min. for *B. subtilis* 4NK. It was determined that the enzyme preserved its activity at a rate of 99% up to 100% for 120 min, and maintained its activity at a rate of 87% up to 120 min at 40 and 50 °C.

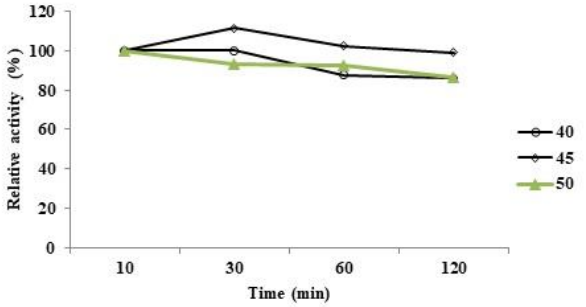

Figure 4. Thermal stability of the 4NK β-galactosidase (The enzyme was incubated at 40-50 °C for different time-course experiments (10-120 min). The unheated purified enzyme was taken as 100%. The remaining β-galactosidase activity was measured under standard assay conditions).

In Figure 5, the thermal stability of β-galactosidase enzyme activity was examined at 45-60 °C in the range of 10-120 min for *B. paralicheniformis* 5NK and the thermal stability of the enzyme was observed at 45-50 °C for 30 minutes. 65% residual enzyme activity was maintained after 120 min at 55 °C. However, enzyme activity was very low at 60 °C Chakraborti et al. (2003), in their study on the β-galactosidase enzyme of thermophilic *Bacillus polymxia*, found that the enzyme preserved its thermal stability at 50 °C, but could not maintain thermal stability at higher temperatures. Also, Levin & Mahoney (1981), as a result of their research on the β-galactosidase enzyme of *Bacillus coagulans* L4, found that this enzyme lost 70% of its enzyme activity in the 30th min at 55 °C.

Table 1. Purification steps of β-Galactosidase

Bacteria	Purification steps	Total Protein (mg/ml)	Total Activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
***B. subtilis* 4NK**	Crude extract	73.3	5328.1	72.7	1	100
	Ammonium sulphate precipitation and dialysis	22.7	4540.1	200.1	2.8	85.2
***B. paralicheniformis* 5NK**	Crude extract	184.7	11399.9	61.7	1	100
	Ammonium sulphate precipitation and dialysis	22.7	8756.9	121.0	2.0	76.8

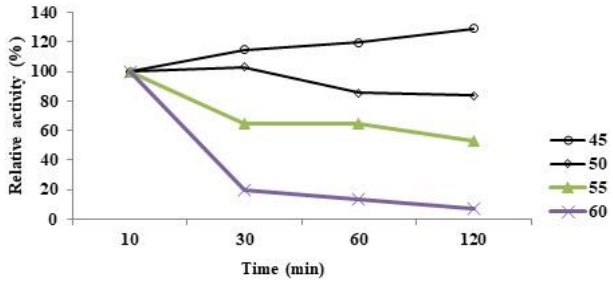

Figure 5. Thermal stability of *the* 5NK β-galactosidase (The enzyme was incubated at 45-60 °C for different time-course experiments (10-120 min). The unheated purified enzyme was taken as 100%. The remaining β-galactosidase activity was measured under standard assay conditions).

Determination of optimum pH

The effect of pH varying between 4.0-11.0 on β-galactosidase activity was investigated. As seen in Figure 6, the optimum pH value was found to be 6.0 for *B. subtilis* 4NK and *B. paralicheniformis* 5NK. Both enzymes from 4NK and 5NK strains were found to be stable at pH 6.0 up to 120 minutes at 45 and 50 °C (data not shown). The optimum pH value of β-galactosidase was found to be 6.0 for *B. subtilis* (Torres and Lee, 1995), *B. circulans* (Vetere & Paoletti, 1998), *B. licheniformis* (Phan Trân et al., 1998), *A. acidocaldarius* subsp. rittmannii (Gul Guven et al., 2007) and *B. licheniformis* KG9 (Matpan Bekler et al., 2015) and it was similar to our study. The pH result shows that its products can be used in milk and milk processes such as processing and lactose hydrolysis.

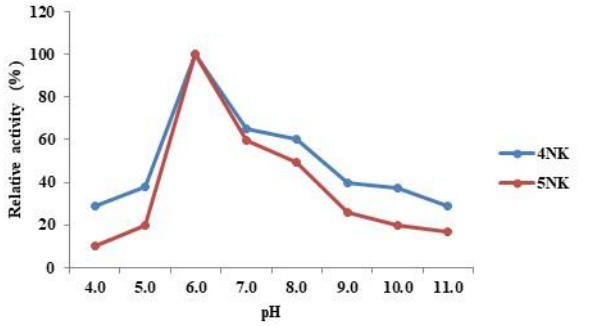

Figure 6. Effect of pH on β-galactosidase (Optimum pH was determined under standard assay conditions using *o*NPG (pH 4.0–10.0)).

Effect of some chemicals and metals on enzyme activity

As can be seen in Table 2, the effects of some chemicals on β-galactosidase activity were examined. It was determined that partially purified enzyme activity increased 13% with 1 mM IAA and 2% with 5 mM PMSF, and increasing concentrations of DTT, NEM and PCMB decreased the enzyme activity for *B. subtilis* 4NK. β-galactosidase activity increased 18% in the presence of 10 mM DTT, 3% with 1 mM NEM and 1% with 5 mM PMSF, and increasing concentrations of IAA, PMSF, NEM and PCMB caused high inhibition for *B. paralicheniformis* 5NK. It is known that the cysteine (Cys) residue is modified by PMSF and NEM and IAA is the SH group alkylating reagent. It can be said that the active center of the enzyme contains Cys and SH group for *B. subtilis* 4NK and *B. paralicheniformis* 5NK since increasing concentrations of PMSF, NEM and IAA inhibit the enzyme. PCMB is an inhibitor of the Cys amino acid and for both bacteria all concentrations of PCMB inhibited the enzyme activity. Considering this information, it is thought that PCMB blocks the sulfhydryl group in the enzyme's active site. In previous studies, Gul Guven et al. (2011), Matpan Bekler et al. (2018) and Shaikhan et al. (2020) stated that PCMB inhibit enzyme activity. Since DTT, which is a thiol marker, does not seriously affect the enzyme, it can be said that thiol groups are not determinant in enzyme activity. The study conducted with *Anoxybacillus* sp. FMB1 (Yalaz et al., 2019) showed similar results. It can be said that the Cys amino acid is present as the proton donor in the active center of the enzyme and it plays a role in breaking the glycosidic bond in the catalytic mechanism.

Table 2. Effect of some chemicals on enzyme activity (retained activity %)

Chemicals	Bacteria	1 mM	5 mM	10 mM
IAA	4NK	113	103	79
	5NK	73	25	14
DTT	4NK	89	72	68
	5NK	111	117	118
PMSF	4NK	88	102	78
	5NK	82	101	35
NEM	4NK	82	91	80
	5NK	103	64	61
PCMB	4NK	17	14	14
	5NK	9	8	7

As seen in Table 3, the activity of β-galactosidase enzyme was investigated on some metal and EDTA as a chelator. The enzyme activity increased in the presence of 1 mM Co^{2+}, 2.5 mM Cu^{2+} and Mn^{2+}, 5mM Mg^{2+}, Zn^{2+} and Cd^{2+}, 10 mM Ni^{2+} and 20 mM Ca^{2+} and EDTA while Zn^{2+}, Cu^{2+}, Mn^{2+} and Cd^{2+}inhibit the enzyme activity at 10 mM and 20 mM for *B. subtilis* 4NK. This table also shows that the enzyme activity increased in the presence of 1 mM Zn^{2+}, Co^{2+}, Ni^{2+} and EDTA, 2.5 mM Ca^{+2}, 5 mM Mg^{2+} and Mn^{2+} while increasing concentrations of Zn^{2+}, Cu^{2+} Ni^{2+} and Cd^{2+} inhibited the enzyme activity for *B. paralicheniformis* 5NK. It should also be taken into consideration that the divalent cations may bind to citrate used to adjust pH to the optimum, which in turn may change the metal sorption distribution coefficient (Kd) values reducing the concentrations of free ions (Lacal et al., 2010).

Generally, β-galactosidases can be activated or inhibited by metal ions or other reagents (Shaikh et al., 1999). In previous studies, Ladero et al. (2002), Shipkowski & Brenchley (2006) and Gul Guven et al. (2011) stated that Cu^{2+} inhibit enzyme activity. It is known that enzyme activity increases with Ca^{2+} and Mg^{2+} in most β-galactosidases (Berger et al., 1997; Ohtsu et al., 1998; Lu et al., 2007; Shaikhan et al., 2020). These

Table 3. Effect of Some Metals and EDTA on Enzyme Activity (retained activity %)

Chemicals	Bacteria	1 mM	2.5 mM	5 mM	10 mM	20 mM
Mg^{+2}	4NK	113	115	135	113	-
	5NK	122	119	136	129	112
Ca^{+2}	4NK	119	116	142	148	149
	5NK	119	126	104	103	-
Cu^{+2}	4NK	143	171	141	116	24
	5NK	15	14	13	15	-
Zn^{+2}	4NK	112	117	124	63	34
	5NK	128	106	110	92	60
Mn^{+2}	4NK	189	193	152	94	49
	5NK	150	160	165	162	123
Co^{+2}	4NK	156	151	154	144	162
	5NK	132	127	128	121	22
Ni^{+2}	4NK	123	148	170	179	165
	5NK	129	114	108	50	-
Cd^{+2}	4NK	-	-	109	39	29
	5NK	94	58	18	18	-
EDTA	4NK	143	153	-	151	162
	5NK	145	124	124	120	-

results support our study. EDTA has been observed to activate the enzyme at all concentrations. Therefore, it can be said that this enzyme is not a metallo enzyme.

Enzyme kinetics

In Figure 7, the *Km* value of the enzyme dependent on *o*NPG concentration for *B. subtilis* 4NK was calculated as 23.80, *Vmax* values as 1.978 µmol/min, and the *Km* value for *B. paralicheniformis* 5NK as 5.61, and *Vmax* values as 1.869 µmol/min. Levin & Mahoney (1981) found the *Km* value for this enzyme as 4.2-5.6 mM as a result of the research on β-galactosidase enzyme of *Bacillus coagulans* L4. *Km* value for *o*NPG counts were 1.32 mM (Shaikh et al., 1999). O'Connell & Walsh (2008) stated that *Km* and *Vmax* values for *o*NPG were 2.23 and 0.56 mM. In addition, O'Connell & Walsh (2010) reported *Km* value was 1.74 mM and V_{max} was 137 UI/mL for *o*NPG.

Conclusion

In this study, β-galactosidase was isolated and characterized from *B. subtilis* 4NK and *B. paralicheniformis* 5NK obtained from Binkap hot water spring in Bingöl province. It was determined that the enzyme was produced in a short time with low cost and high efficiency, and also the pH and temperatures of enzymes were suitable for milk and dairy industries and that they showed stability. In addition, β-galactosidase is the first in enzyme studies regarding *B. paralicheniformis* with this study.

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Author Contributions

ŞT: Conceptualization, Writing-review and editing; FMB: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing-original draft; KG: Funding Acquisition, Project Administration, Resources, Writing-review and editing.

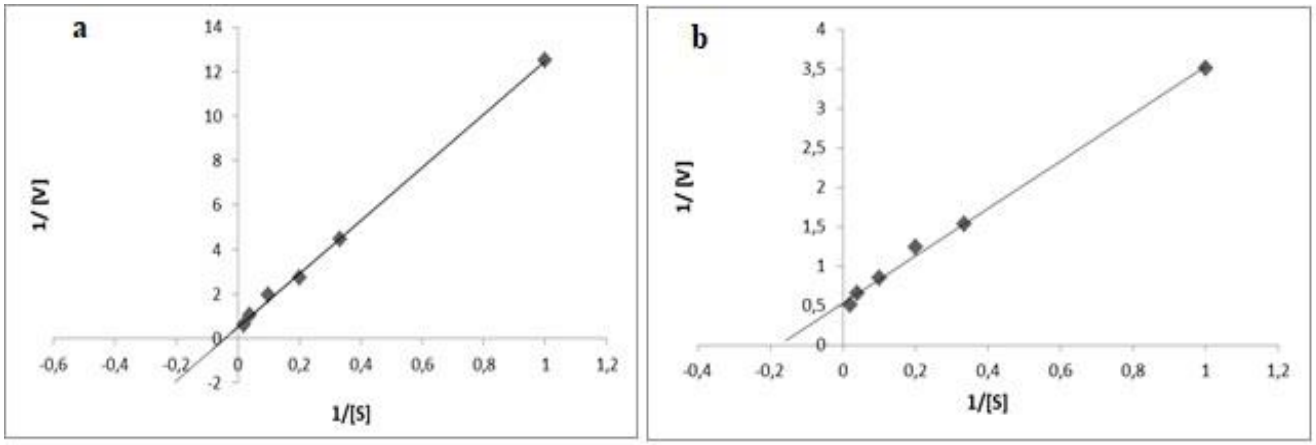

Figure 7. Linewear-Burk for **a)** *B. subtilis* 4NK and **b)** *B. paralicheniformis* 5NK.

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RESEARCH PAPER

Genome-wide analysis of *Fragaria vesca* Three-Amino-Acid-Loop-Extension (TALE) genes

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Abstract

The present study is aimed to identify and characterize the three-amino-acid-loop-extension (TALE) genes in *Fragaria vesca* as bioinformatics. TALE superclass homeoproteins have important roles in regulating certain signal pathways in the plant system. However, there is no knowledge about the role of *TALE* genes in *Fragaria vesca*. According to this study, a total of 18 candidate *FvescaTale* genes were identified. Identification of motifs, exon and intron analysis, genome mapping, localization in the cell, three-dimensional modeling, and ontology analysis were made according to these genes. This bioinformatic analysis revealed that *FvescaTale* genes might play an important role in stress response for *Fragaria vesca* cultivars and suggests that these genes could be used as functional markers for *in silico* analysis for future studies.

Introduction

Fragaria vesca, a diploid (2n=14) type of strawberry, is a plant that belongs to the Rosaceae family (Li et al., 2019; Shulaev et al., 2011). *Fragaria vesca* is a model used in this family due to its ease of proliferation, small genome length, and short generation cycle compared to other plants. Despite all these qualities of *Fragaria vesca*, its genome was sequenced and revealed in 2010 (Shulaev et al., 2011). *Fragaria* has not a real fruit, it consists of seeds that modified the surface of the reservoir formed at the tip of the shoot. Strawberry, which bred from four different diploid ancestors, is important in genomic research both phylogenetically and because it has a small genome. It has an extensive genotypic diversity thanks to its optimum growth range, vegetative reproduction and many positive recessive properties. Therefore, it is an important plant to determine the genomic characteristics of the Rosaceae family (Darrow, 1966; Shulaev et al., 2011).

A homeobox (HB) encodes a homeodomain (HD) region that is 60 amino acids in length and interacts with DNA specifically. This region is conserved in transcription factors (TF) in all eukaryotic organisms (Ariel et al., 2007). The protein class of homeoproteins called the three-amino-acid-loop-extension (TALE) has been shown to direct organ morphogenesis, meristem continuity or formation, various properties of the reproductive phase, and organ orientation (Hamant & Pautot 2010). Homeobox proteins have been shown to have a TALE superclass and are recognized by an extension of three amino acids (Pro-Tyr-Pro) between α-helices 1 and 2 in the homeodomain. Genes encoding these proteins are highly conserved. It has been observed that plants, fungi, and animals also have transcriptional regulatory functions in their common ancestors and they are crucial for signaling and communication network (Chen, 2003; Burglin, 1997).

Knotted 1-like homeobox (KNOX) proteins, a homeodomain transcription factor, control genes that regulate hormone homeostasis in the shoot apical meristems of plants. KNOX genes contain the TALE

homeobox gene family, which acts as a regulator in the diploid development of plants (Hay & Tsiantis 2010). Homeobox genes play an important role in transcriptional regulation in various plants, shoot apical development and flowering, lignin and cellulose accumulation, cell wall biosynthesis, plant growth and development under high temperature and humidity stress (Rutzens et al., 2009; Hirano et al., 2013; Liu et al., 2014). There are some studies on TALE members, but functional genomic studies are lacking in most plants. Therefore, elucidating the function of the TALE family in plants provides an important genomic resource for genome-wide analysis (Ma et al., 2019).

The aim of study is to define and characterize the TALE family, one of the genes to be used at the transcriptional level in future biotic and abiotic stress studies to be carried out with *Fragaria vesca*. Thus, the genomic functions of the relevant genes will be known and the results will be evaluated *in silico*. This study is an important preliminary resource that includes many bioinformatic analysis for future studies. According to this research, genome-wide analyzes were performed for *F. vesca* TALE genes using various bioinformatics tools. As a result, it is suggested that these genes can be used as functional markers *in silico* analysis for future studies.

Materials and Methods

Identification of TALE genes in *Fragaria vesca*

F. vesca TALE protein sequences were retrieved from Phytozome database v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) using keywords in the search with Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/) and Pfam Database (http://pfam.xfam.org/). To identify TALE proteins in the *F. vesca* genome, both blastp at Phytozome database v12.1 (http://www.phytozome.net) and Hidden Markov model (HMM), (http://www.ebi.ac.uk) searches were performed against the *F. vesca* genome.

The solid and chemical traits of TALE proteins in *F. vesca* were identified using the ProtParam tools (http://web.expasy.org/protparam/) such as: molecular weight, atomic composition, extinction coefficient, theoretical pI, estimated half-life, amino acid composition, aliphatic index, instability index and grand average of hydropathicity.

TALE proteins of identification of motif paterns, locations, and 3D modelling

Motifs present in the TALE protein families were identified by using the Multiple Expectation Maximization for Motif Elicitation (MEME) tool (http://meme-suite.org/). Protein is classified by families, predicting domains, and important sites by using Interpro Tool (https://www.ebi.ac.uk/interpro/).

Three Dimensional (3D) Structures for all the proteins were reported by using Phyre2 Tool. (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) Phyre2 is also used for functions and mutations.

Phylogenetic analysis, physical, chromosomal location

Phylogenetic analysis was reported by MegaX tool for 18 TALE genes. Gene Structure Display Server program tool (GSDS; http://gsds.cbi.pku.edu.cn/) was used to estimate the exon/intron set up of the TALE genes. The location of the genes on the chromosome was determined by using MapGene2 tool (http://mg2c.iask.in/mg2c_v2.0/).

Examination of *cis*-regulatory elements in the promoter region and subcellular localization

2 kb sequences in the upstream region of the 18 genes were analyzed in the PlantCARE program (http://sphinx.rug.ac.be:8080/PlantCARE/).

WoLF PSORT tool was used to analyze where TALE genes are located in the model organism (https://wolfpsort.hgc.jp/). WoLF PSORT converts amino acid sequences of a protein into numerical localization properties so the results show where genes are located.

Plant small RNA target analysis

psRNATarget was used for the relationship between the transcript of the gene and the miRNA targeted for silencing. The psRNATarget analysis program includes the latest innovations in miRNA target recognition in the plant. This program was used to display sRNA targets in the plant by finding the match found between the sRNA sequence and the target mRNA sequence (https://plantgrn.noble.org/psRNATarget/).

Ontology analysis

Ontology analysis, biological and molecular functions were reported by using AgriGO tool. It is a major bioinformatics initiative to combine gene and gene product characteristics across all species. This tool supports special focus on agricultural species. (http://bioinfo.cau.edu.cn/agriGO/)

Results

Analysis of TALE gene family in *Fragaria vesca*

As a result of screening and profiling the TALE gene from the existing protein databases, the presence of eighteen proteins for *Fragaria vesca* were found. The protein sequences of these genes were obtained from Phytozome database v12.1. Firstly, these sequences were analyzed by ProtParam. The results were given in Table 1. Accordingly, the amino acid length of the *FvescaTale.1* gene is found to be 670. This gene is 73882.80 kDA, and it has 5.67 pI. This gene is unstable and the instability index is 48.65. According to our data, only *FvescaTale.14* gene is found stable with 38.27 instability index.

Table 1. Information of TALE genes in Fragaria vesca along with their gene codes, number/length of amino acids, mass (kDa), pI, stability and instability index

Gene Code	Amount of Amino acid	Mass (kDa)	pI	Stability	Instability Index
FvescaTale.1	670	73882.80	5,67	unstable	48.65
FvescaTale.2	809	88184.05	6,04	unstable	43.11
FvescaTale.3	662	74135.92	5,41	unstable	49.79
FvescaTale.4	598	66747.32	6,91	unstable	56.50
FvescaTale.5	399	44532.13	6,28	unstable	49.14
FvescaTale.6	815	87527.38	6,73	unstable	53.86
FvescaTale.7	216	24764.15	6,31	unstable	48.22
FvescaTale.8	406	46114.01	8,85	unstable	54.04
FvescaTale.9	795	88721.63	5,58	unstable	52.68
FvescaTale.10	318	35355.75	8,70	unstable	59.47
FvescaTale.11	470	52130.66	6,79	unstable	43.53
FvescaTale.12	933	103801.21	7,86	unstable	39.27
FvescaTale.13	477	54344.88	7,74	unstable	48.12
FvescaTale.14	330	37277.88	5,04	stable	38.27
FvescaTale.15	184	20981.38	5,66	unstable	60.73
FvescaTale.16	391	44500.16	5,62	unstable	41.34
FvescaTale.17	323	36600.06	5,48	unstable	48.13
FvescaTale.18	289	32623.65	6,24	unstable	57.46

Identification and domain relationships of motifs in the TALE family in *Fragaria vesca*

Using MEME program, eighteen motifs were identified by predicting the motifs of TALE genes in the *Fragaria vesca* family. The motifs of the seven genes between the *FvescaTale.12* and *FvescaTale.18* are colorless unlike the other motifs. This is due to the lack of connection between homologs and the irregularity of motifs. In addition, motif locations are checked over MEME Suite.

The domain relationships of the motifs were analyzed via InterProScan program. Figure 1 has showed eleven TALE proteins logo patterns. The other proteins have unstable sequences. They have been looked uninspiring as a result of MEME analysis. All logo patterns have diffirent aminoacid configurations (Figure 1). But most of them contain similar consensus sequence.

When Figure 2 is examined, it is observed that FvescaTale.1, FvescaTale.2, FvescaTale.3 and FvescaTale.4 motifs have homology similarity. Likewise, similar motifs were observed in FvescaTale.16, FvescaTale.17 and FvescaTale.18 due to the presence of domains in close proximity (Figure 2).

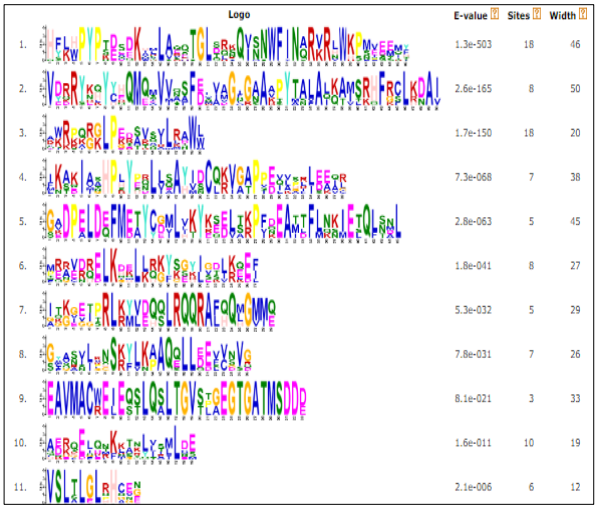

Figure 1. Motif sequences analysis in amino acid sequences of TALE from *Fragaria vesca.*

Exon and intron analysis of TALE gene family in *Fragaria vesca*

In the Supplementary data 1, the yellow areas indicate exons, while the black lines indicate introns. The positions and numbers of the exon and intron regions on the TALE genes of *Fragaria vesca* were shown.

This analysis was carried out by separating exon and intron from GeneStructure Display. The importance of exon and intron number gives the relationship between genes. Four exons and three introns are found in the *FvescaTale.8* gene, eleven exons and ten introns are found in *FvescaTale.12* and finally, five exons and four introns are found in the *FvescaTale.18* gene. The

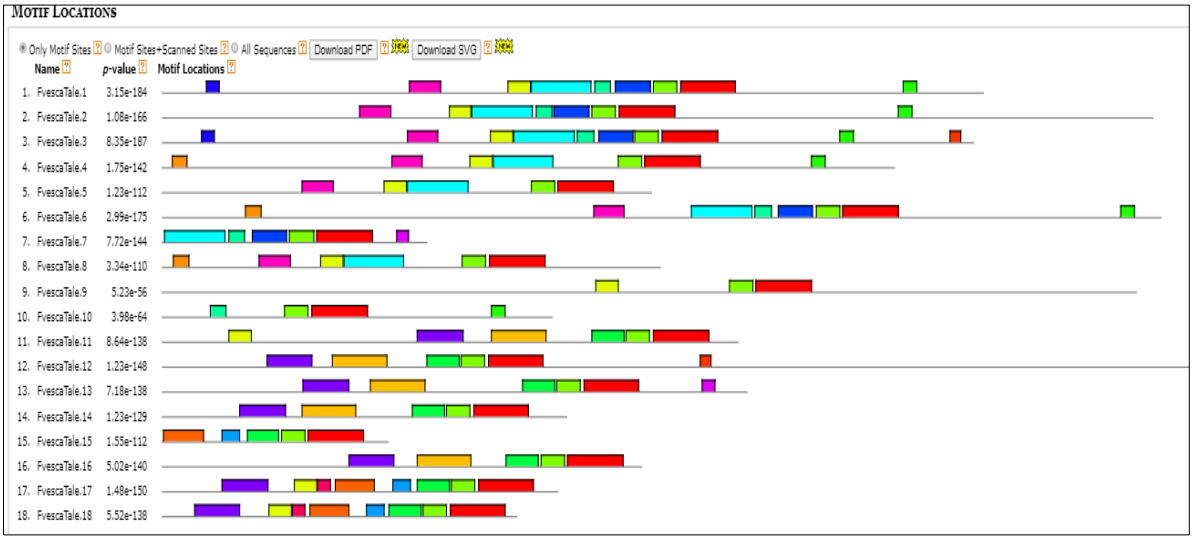

Figure 2. Motif locations analysis in amino acid sequences of TALE from *Fragaria vesca.*

importance of intron numbers is still controversial. However, the length of existing introns indicates that the gene region will be stable and conserved. Sequences of preference for gene expression and RNAseq analysis e with long introns. *FvescaTale.2, FvescaTale.12* and *FvescaTale.16* have long intron regions (Supplementary data 1).

Genome mapping of TALE gene family in *Fragaria vesca*

The studies on MapGene2 were shown in Figure 3. Eighteen TALE transcription factors identified on the 7 chromosomes of *F. vesca* were distributed. According to these distributions, *FvescaTale.17* and *FvescaTale.14* genes were found on chromosome 1. *FvescaTale.1, FvescaTale.15*, *FvescaTale.9, FvescaTale.3* and *FvescaTale.5* genes were localized on chromosome 2. Chromosome 3 contained the *FvescaTale.11* and *FvescaTale.20* genes. *The FvescaTale.16* gene was found on the chromosome 4.

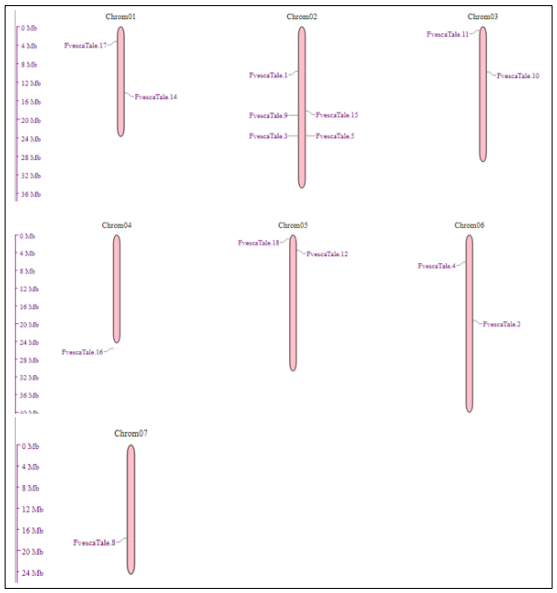

Figure 3. Genome mapping by positioning the TALE family on chromosomes.

The FvescaTale.18 and *FvescaTale.12* genes were observed on the chromosome 5. *FvescaTale.4* and *FvescaTale.2* are located in the chromosome 6. The *FvescaTale.8* gene was localized on the chromosome 7. *Fragaria vesca* has small genome, but it appears that genes are largely distributed on different chromosomes. This is proof that it is important evolutionarily.

Distribution of TALE gene family in *Fragaria vesca* in the cell

The distribution of Tale genes in *Fragaria vesca* in the cell is given in Table 2. Cellular localizations of the eighteen genes belonging to *F. vesca* were evaluated by WoLF PSORT. According to Table 2, 7.5 of the *FvescaTale.2* gene were found in the cytoplasm and 13.5 were found in the nucleus. Fourteen of the *FvescaTale.5* gene were localized in the nucleus. While thirteen of the *FvescaTale.10* gene were in the nucleus and one in the chloroplast. Thirteen of the *FvescaTale.14* gene were observed in the nucleus and one in the peroxisome (Table 2).

Table 2. Distribution of TALE family in *Fragaria vesca* in the cell compartments

Gene names	Cytoplasm	Nucleus	Peroxisome	Chloroplast
FvescaTale1		14		
FvescaTale2	7.5	13.5		
FvescaTale3	7.5	13.5		
FvescaTale4		14		
FvescaTale5		14		
FvescaTale6	7.5	13.5		
FvescaTale7		13	1	
FvescaTale8		14		
FvescaTale9		13	1	
FvescaTale10		13		1
FvescaTale11	7.5	13.5		
FvescaTale12		14		
FvescaTale13	7.5	13.5		
FvescaTale14		13	1	
FvescaTale15		14		
FvescaTale16		14		
FvescaTale17		14		
FvescaTale18		14		

3D modeling of TALE proteins in *Fragaria vesca*

The 3D motifs of the eighteen TALE proteins studied were analyzed using the Phyre2 program. These results were shown in Figure 4. According to the data, 38% of the remnants of FvescaTale.5 were modeled with >90% confidence. It was estimated that 54% of the series are irregular (Figure 4).

Ontology analysis of TALE gene family in *Fragaria vesca*

The results of AgriGO are attached in the form of a table in the Supplementary data 2. AgriGO database is used to determine ontology analysis of genes. According to Figure 5, 80% of our genes participate in cellular processes while 15% have function. Together with these, 5% function as a cellular components (Supplementary data 2).

Phylogenetic analysis of Tale proteins family in *Fragaria vesca*

In the MegaX program, relationships between 18 TALE proteins were determined. According to the result shown in Figure 6, the relationship between FvescaTale.14 and FvescaTale.5 proteins were remote, while FvescaTale.11 and FvescaTale.1 were closely related.

Eighteen TALE proteins belonging to *F. vesca* organism and *TALE* proteins found in *O. brachyantha* and *F. ananassa* organisms were compared. According to Figure 7, FvescaTale.11 and FananassaTale.8 were closely related. FananassaTale.6 and ObrachyanthaTale.15 have been observed in distant association.

The eighteen TALE genes from the *F. vesca* organism were compared with miRNA sequences from other plant species via psRNATarget. The results are given in the Supplementary data 3.

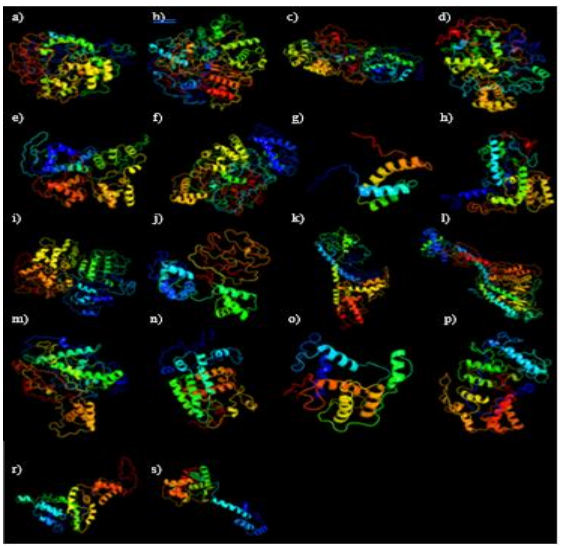

Figure 4. 3D modeling of TALE proteins a)FvescaTale1, b)FvescaTale2, c)FvescaTale3, d)FvescaTale4, e)FvescaTale5, f)FvescaTale6, g)FvescaTale7, h)FvescaTale8, i)FvescaTale9, j)FvescaTale10, k)FvescaTale11, l)FvescaTale12, m)FvescaTale13, n)FvescaTale14, o)FvescaTale15, p)FvescaTale16, r)FvescaTale17, s)FvescaTale18. Image coloured by rainbow N → C terminus.

According to the data, 1759 relationships were found (Supplementary data 3). Two of the *FvescaTale.4* genes were associated with the *Arabidopsis thaliana* miRNA. Similarly, 3 of the *FvescaTale.14* genes and one of the *FvescaTale.16* gene were associated with *Oryza sativa*.

Discussion

In this present study, a total of eighteen candidate *FvescaTale* genes were identified. While the lowest number of *FvescaTale* genes was on chromosomes 4 and 7 (one Tale gene), the highest number of *FvescaTales* was on chromosome 2 (five TALE genes). One gene, *FvescaTale.7,* was found to have no-introns. *FvescaTale.12* had the highest number of introns (10 introns).

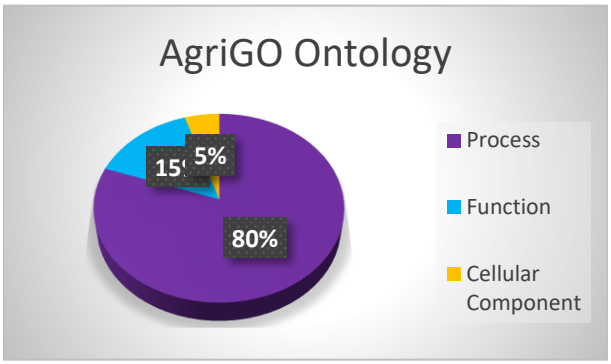

Figure 5. AgriGO Ontology graphical datas.

Even if the intron number does not give clear information, the lengths of the introns are important. In a possible gene expression, gene regions with long introns are preferred. Because it is more stable. In addition, all TALE genes were found in the nucleus, while *FvescaTale.2, 3, 6, 11* and *13* were found in the cytoplasm. Moreover, studies have been carried out on the 3-dimensional structures and ontology analysis of these genes. Phylogenetic studies of these 18 genes have been carried out and comparisons with different organisms have also been made. When the motif patterns of the proteins identified according to the results were compared, it was seen that the members with similar motif patterns were located in a close cluster on the phylognetic tree. Although protein domains are not characterized by a known family, it has been observed that the figures obtained have similar motif locations with the beta helix loop helix. The fact that the 18 TALE proteins have different motif patterns indicates that the *cis*-regulatory elements in the upstream region are very different. Phylogenetic tree data also support this hypothesis. Because each tree branch is clustered at a certain distance from each other.

After the whole genome sequence analysis is done, definitions and characterizations should be made on certain parts of the genome in order to determine the

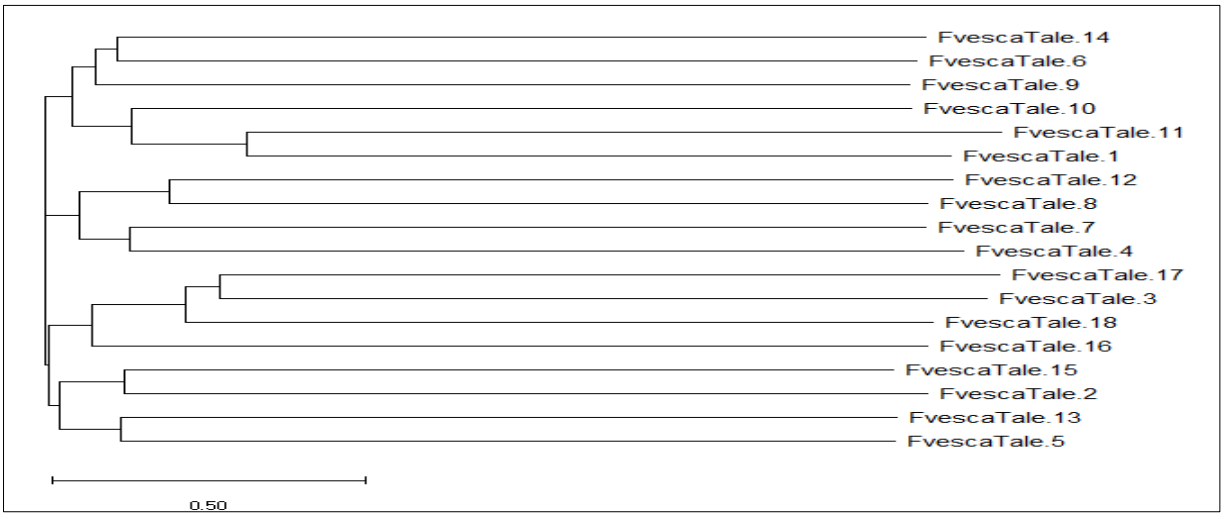

Figure 6. Phylogenetic tree of TALE proteins.

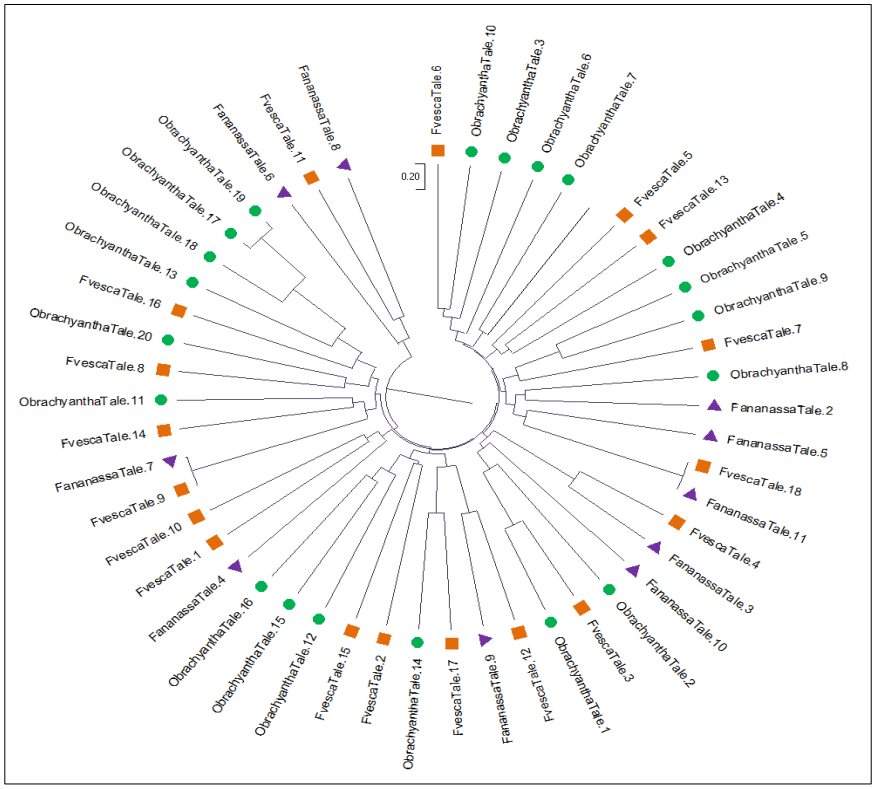

Figure 7. Phylogenetic circle tree of TALE proteins in *F. vesca, O.brachyantha* and *F.ananassa.*

genomic features functionally. Compared to existing studies, the data obtained allows us to infer whether the analyzed region of the genome is involved in any stress-related pathway. For example, after the genome sequences of 4 different cotton species were completed, the development of functional genomic analyzes of cotton was proposed and the genome-wide characterization of the genes of the TALE family was made. Following this process, it has been demonstrated that *TALE* genes regulate secondary cell wall synthesis. Unlike *F. vesca*, 46, 47, 94 and 88 *TALE* genes were identified, respectively (Ma et al., 2019).

TALE homeobox genes are an important group of genes that play a role in developmental processes in both plants and animals. It is also expressed from time to time in the early development stages of some living things. A study conducted on Spiralians has also been shown to play an important role in the cellular separation mechanism (Morino et al. 2017). Since we know that these genes are expressed along the plant-animal line, *TALE* genes are also very important for the evolutionary process. Especially, thanks to common *cis*-regulatory elements found in both animals and plants, genomic inference can be made. Related the elements have some common functions such as transcriptional regulation, *cis*-regulator regions, stress-related processes, functional processes, circadian rhythm processes and etc. Therefore, it enables inferences in plant-animal interaction and genome-wide association studies.

It is not known whether *TALE* genes, which are expressed in almost every living organisms, arise from a single ancestor or independently of the ancestral *TALE* genes. Therefore, it is necessary to determine the role of *TALE* genes in both plant and especially stress response pathways and plant-animal interactions with common conserved protein domains and *cis*-regulatory elements. Identification and characterization of *TALE* genes at the stages of the evolutionary process at the species and subspecies level will at least provide an elucidation of the mechanism in the pathways that respond to stress.TALE homodomains contain polar residues such as glutamine, lysine, cysteine, histidine or serine. These small polar residues show that the DNA-Protein interactions of *TALE* genes are very different. This proves the existence of a species-specific transcriptional arrangement. Conserved motifs are found between TALE and other homodomain genes in similar pathways. The ELK region in the *Zea mays* plant is an example. In addition, at least 1 intron position is conserved within the KNOX genes. There is a protected GSE-box area within the KNOX and ELK area (Akam 1993).

The determined intron positions do not shed much light on the evolutionary distinction, but indicate that genes belonging to two different subclasses in the TALE family, such as KNOX and BEL, which are expressed in similar pathways and have the same homodoma, may be expressed at different levels in different organisms. This shows that these genes are actually different gene groups specific to each organism (Reiser et al. 1995; Bürglin 1997). This situation shows the importance of genome-wide characterization and identification studies.

In silico analysis enables understanding of DNA-protein interactions. Here, we made the genome-wide *in silico* analysis of the *Fragaria* and *TALE* genes, which is a plant species. Although it gives us limited information on the evolutionary process, we were able to obtain important reference data for plant-plant or plant-animal interreactions. For *in-vivo* analyzes to be performed with *TALE* genes in the future, data defined at the transcriptional level will be available. In order to determine the efficiency of *TALE* genes in stress-related pathways, *in silico* analysis of the relevant genes should be done before qrt PCR studies. Therefore, this study is a preliminary study to understand transcriptional regulation at the level of gene expression. Besides, this bioinformatic analysis revealed that *FvescaTale* genes might play an important role in stress response for *Fragaria vesca* cultivars and suggests that these genes could be used as functional markers for *in silico* analysis for future studies.

Conclusion

In summary, this study provides important clues for further elucidating the functions of *TALE* genes regulating and development in *Fragaria vesca*. In future studies, researchers could use this information to correlate results regarding gene expression. Therefore, this study is part of the genome-wide identification and characterization step *in silico*. The characteristics of the TALE homeodomain family in *F. vesca* have been determined bioinformatically.

Author Contributions

Conceptualization Ideas; FŞG, Data Curation; GK, SŞ, ST, FŞG, Formal Analysis; GK, SŞ, ST, FŞG Investigastion; GK, SŞ, ST, FŞG, Methodology: GK, SŞ, ST, FŞG, Validation; GK, SŞ, ST, FŞG, Visualization; GK, SŞ, ST, FŞG, Writing-original Draft Preparation; GK, SŞ, ST, FŞG.

Additional Information

Supplementary data accompanies this paper at https://biotechstudies.org/uploads/BIO-126_Supp1.pdf https://biotechstudies.org/uploads/BIO-126_Supp2.pdf https://biotechstudies.org/uploads/BIO-126_Supp3.pdf

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RESEARCH PAPER

Effect of different concentration of exogenous proline applications on cadmium accumulation and mineral nutrition (K, Mg, Na, and Ca) of common wheat (*Triticum aestivum*)

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Abstract

The present study investigates the role of exogenously applied proline on cadmium (Cd) accumulation in common wheat (*Triticum aestivum* L.) tissues. Seedlings were subjected for 4 days to different exogenous proline levels (0, 1, 10, and 20 mM) under Cd stress (1000 µM of $Cd(NO_3)2{\cdot}4H_2O$). The concentration of Cd, Ca, Mg, and K was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Exogenous proline caused significant changes in the growth of wheat cultivar under Cd stress. In addition, the growth of wheat under Cd stress increased by the addition of 1 mM proline. According to the analysis results, Cd accumulation in wheat seedlings showed that the increment of exogenous proline treatments (except Cd+Pr1) in the water resulted in a decrease of Cd content in roots and shoots. Under Cd treatment (not applied proline), the contents of Ca, K, Mg, and Na decreased in roots by 22.1, 70, 17.7, and 10.1% and in shoots by 29.6, 32.2, 19.1, and 5.3%, respectively. Nevertheless, K content decreased in roots and shoots under all Cd and exogenous proline treatments.

Introduction

Plants are affected by environmental factors such as toxic metal accumulation, temperature extremes, radiation, and excessive salinity throughout their life cycle. Growth retardation or inhibition can also occur when plants are exposed to abiotic stress factors such as toxic metals concentration as (Çatav et al., 2020).

Toxic metals such as arsenic (As), lead (Pb), and cadmium (Cd) which are increasing in nature due to human activities, represent a significant threat to plants. Exposure of plants to excessive metal concentrations may cause structural and physiological disturbances. Environmental contamination by Cd occurs in many countries as a result of intensive use of agrochemical, anthropogenic and industrial activities such as mining and plastic manufacturing. In addition, Cd can remain in nature for decades.

Since Cd causes many diseases, its entry into the food chain should be minimized and new strategies should be developed. However, a long time is needed to produce food containing low amounts of Cd. In addition, due to its high mobility in the soil, the consumption of plants grown in soils with high Cd accumulation may pose serious threats to human and animal health. Cd causes damage even at low concentrations. Cd damages the kidneys and also causes osteoporosis by inhibiting calcium uptake and vitamin D activation (Jarup et al., 1998). Cd enters through roots in plants, impairing nutrient accumulation and restricts plant growth and it also damages the photosynthesis system (Bashir et al., 2018).

The consumption of grains is one of the main sources of Cd. Reducing Cd accumulation in wheat is crucial since it is the third most consumed grain in the world. Therefore, in this study, wheat was chosen to

discuss the exogenous application of Cd and its uptake in plants. Approximately, 60% of the population in developing countries consumed the wheat as a staple food. Due to the increase in the world population, the demand for wheat is increasing and it is expected to rise by 70% in the next few decades (Vitale et al., 2020).

Exogenous applications are an effective and fast method to reduce Cd toxicity in plants. Proline accumulation in plants is an adaptive mechanism that occurs under stress conditions of the plant. However, it has been suggested that the accumulation of proline increases the tolerance of most species to stress conditions such as toxic metals (Islam et al., 2009). Some scientists consider proline as an essential amino acid to reduce metal stress while others consider it a response to stress accumulation (Ashraf & Foolad, 2007). In addition, the natural amount of proline in the plant may not be sufficient to protect it from adverse effects of Cd stress (Okuma et al., 2002; Tamura et al., 2003; Tamas et al., 2008). Proline protects plants from denaturation of enzymes and osmotic damage, buffers cytosolic pH, acts as an enzyme protectant, and free radical scavenger (Sharmila & PardhaSaradhi, 2002).

Wheat is an important nutrient consumed worldwide as a staple food. Nevertheless, many abiotic factors affect the yield of the wheat crop, including metal stress such as Cd accumulation. There is limited study in the literature describing the relationship between exogenous proline and Cd accumulation in wheat. Therefore, this study aims to determine to what extent exogenous proline compound can reduce Cd toxicity in wheat.

Materials and Methods

Plant material, growth conditions, and treatments

Wheat seeds (cv. Bayraktar-2000) were sterilized with 3% (v/v) sodium hypochlorite and germinated on sterile filter papers moistened with distilled water at 22 ± 1°C in the dark for 4 days. Similar-sized seedlings were then grown hydroponically under a 16-h photoperiod at 22 ± 1 °C for 3 days as described by Çatav et al. (2020). Treatments were started by adding cadmium nitrate tetrahydrate (CAS No. 10022-68-1, Panreac) and L-proline (CAS No. 147-85-3, Sigma-Aldrich) to the nutrient solutions. A randomized complete block design consisting of one control and four treatment groups was used in this study. Four replicates of 20 seedlings were used for each treatment, and the experiment was repeated 4 times. The experimental study is presented schematically in Figure 1. (i) CP: wheat cultivars untreated with $Cd(NO_3)2{\cdot}4H_2O$ and exogenous proline; (ii) Cd: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$; (iii) Cd+Pr1: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 1 mM exogenous proline; (iv) Cd+Pr10: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 10 mM exogenous proline; (v) Cd+Pr20: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 20 mM exogenous proline. Seedlings from control and treatment groups were grown under the same growth conditions for additional 4 days.

The plants were divided into roots and shoot at harvest, washed with ultrapure water, and dried with filter papers. Root and shoot samples were dried in the oven at 70 °C and then weighed. Finally, the oven-dried plant material was ground in a stainless-steel electric grinder.

Sample preparation and analysis

Ultra-pure water obtained from the Direct-Q® 8 UV ultra-pure water system (Merck Millipore, Darmstadt, Germany was used throughout the study. The Teflon vessel was treated with 5% HNO_3 for more than 48 hours, washed with ultrapure water, and dried at 70 °C. For Cd analysis, approximately 150-300 mg of each sample was placed in a closed Teflon vessel containing 7 mL (65%) HNO_3 acid and 3mL (30%) H_2O_2 (Merck, Darmstadt, Germany). Then, the samples were digested in a microwave digestion system (Berghof Speedwave MWS-3+; Berghof, Eningen, Germany). All chemicals used in the experiments were analytical reagent grade. The digestion flasks were then placed in a microwave digestion unit with a gradual increase in temperature until all samples were dissolved. Microwave digestion programming is shown in Table 1.

After digestion, the sample digests were diluted with 100 mL of ultrapure water and filtered through filter papers (Sartorius-Stedim, particle retention = 2-3µm) then transferred into a 25 ml flask. After filtration, the contents of Cd, sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES Agilent 5100). ICP OES operating conditions are shown in Table 2. The wavelength values (nm) were as follows: Cd (228.804), K (766.490), Mg (285.213), Na (589.592), and Ca (315.887).

Data analysis

One-way ANOVA followed by Tukey's HSD test was used to determine whether Cd, Na, K, Ca, and Mg accumulations differ significantly between roots and shoots, and the significance level for the test was set at $P< 0.05$. The heatmap was conducted using the ggplot2 package in R software. SPSS 20.0 was applied for all statistical analysis while Graphpad Prism 7 was used to draw graphs.

Results and Discussion

Plant growth measurement

Wheat seeds (cv. Bayraktar-2000) were sterilized with 3% (v/v) sodium hypochlorite and germinated on sterile filter papers moistened with distilled water at 22 ± 1°C in the dark for 4 days. Similar-sized seedlings were then grown hydroponically under a 16-h photoperiod at 22 ± 1 °C for 3 days as described by Çatav et al. (2020). Treatments were started by adding cadmium nitrate

tetrahydrate (CAS No. 10022-68-1, Panreac) and L-proline (CAS No. 147-85-3, Sigma-Aldrich) to the nutrient solutions. A randomized complete block design consisting of one control and four treatment groups was used in this study. Four replicates of 20 seedlings were used for each treatment, and the experiment was repeated 4 times. The experimental study is presented schematically in Figure 1. (i) CP: wheat cultivars untreated with $Cd(NO_3)2{\cdot}4H_2O$ and exogenous proline; (ii) Cd: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$; (iii) Cd+Pr1: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 1 mM exogenous proline; (iv) Cd+Pr10: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 10 mM exogenous proline; (v) Cd+Pr20: wheat cultivars treated with 1000 µM of $Cd(NO_3)2{\cdot}4H_2O$ and 20 mM exogenous proline. Seedlings from control and treatment groups were grown under the same growth conditions for additional 4 days.

The plants were divided into roots and shoot at harvest, washed with ultrapure water, and dried with filter papers. Root and shoot samples were dried in the oven at 70 ^oC and then weighed. Finally, the oven-dried plant material was ground in a stainless-steel electric grinder.

Sample preparation and analysis

Ultra-pure water obtained from the Direct-Q® 8 UV ultra-pure water system (Merck Millipore, Darmstadt, Germany was used throughout the study. The Teflon vessel was treated with 5% HNO_3 for more than 48 hours, washed with ultrapure water, and dried at 70 °C. For Cd analysis, approximately 150-300 mg of each sample was placed in a closed Teflon vessel containing 7 mL (65%) HNO_3 acid and 3mL (30%) H_2O_2 (Merck, Darmstadt, Germany). Then, the samples were digested in a microwave digestion system (Berghof Speedwave MWS-3+; Berghof, Eningen, Germany). All chemicals used in the experiments were analytical reagent grade. The digestion flasks were then placed in a microwave digestion unit with a gradual increase in temperature until all samples were dissolved. Microwave digestion programming is shown in Table 1.

Table 1. Microwave digestion program

Step	A	B	C	D	E
Temperature [^oC]	100	120	190	120	100
Pressure [bar]	30	30	30	30	0
Hold Time [min]	4	5	10	5	5
Ramp [min]	5	5	5	1	1
Power [%]	60	80	80	60	0

After digestion, the sample digests were diluted with 100 mL of ultrapure water and filtered through filter papers (Sartorius-Stedim, particle retention = 2-3µm) then transferred into a 25 ml flask. After filtration, the contents of Cd, sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES Agilent 5100). ICP OES operating conditions are shown in Table 2. The wavelength values (nm) were as follows: Cd (228.804), K (766.490), Mg (285.213), Na (589.592), and Ca (315.887).

Table 2. The parameters of the ICP-OES

Parameters	
Power (W)	1,450
Plasma gas flow rate (L min^{-1})	15
Auxiliary gas flow-rate (L min^{-1})	0.2
Nebulizer gas flow-rate (L min^{-1})	0.8
Sample flow rate (L min^{-1})	1.5
Visible mode	Axial-radial
Source balancing time (s)	15
Reading time (s)	60
Replicate	3
Cleaning gas	Argon

Data analysis

One-way ANOVA followed by Tukey's HSD test was used to determine whether Cd, Na, K, Ca, and Mg accumulations differ significantly between roots and shoots, and the significance level for the test was set at $P< 0.05$. The heatmap was conducted using the ggplot2 package in R software. SPSS 20.0 was applied for all statistical analysis while Graphpad Prism 7 was used to draw graphs.

Results and Discussion

Plant growth measurement

Exposure of wheat cultivars to Cd and all exogenous proline treatments resulted in a statistically significant ($P < 0.05$) decrease in the length of roots and shoots. Cd+Pr1 application significantly increased the dry weight of roots compared to the Cd application ($P < 0.05$). Statistical differences ($P < 0.05$) were found in the dry weight of shoots when all treatment groups and control groups were compared. In addition, toxicity symptoms such as chlorosis and root browning occurred in the seedlings treated with Cd and Cd+Pr20. In Cd+Pr20 treated wheat cultivars compared to the control group, the decrease of dry weight in roots, shoots, and total seedling was about 17.41, 16.18, and 16.5% respectively. However, comparing Cd treatment

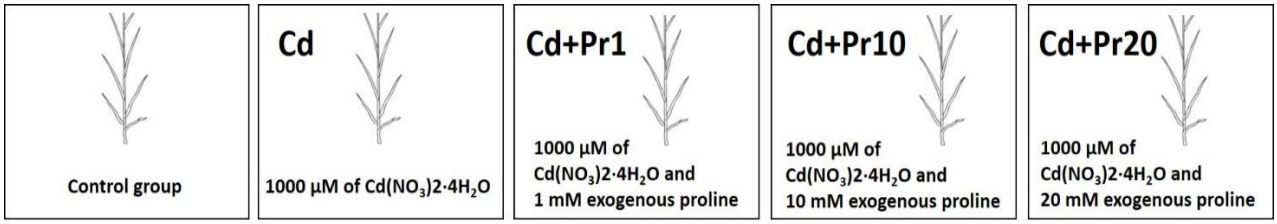

Figure 1. Experimental study of wheat cultivars.

which is not including exogenous proline applications and control treatment, the decrease in dry weight of roots, shoots and total seedling was 24.65, 21.77, and 22.58% respectively (Table 3).

Exogenous proline application in wheat (*Triticum aestivum* L.) alleviated the negative effects on growth and development caused by drought (Kamran et al., 2009). Exogenous proline was applied to corn (*Zea mays*) under the drought stress. The result showed that proline had a positive effect on growth by promoting the uptake of Ca^{+} K^{+}, and N (Islam et al., 2009). Ali et al. (2008) applied exogenous proline as a spray treatment while the corn plant was in the seedling stage. As a result, the significant growth was observed with respect to control group in the environment with water deficiency. Cd stress applied to wheat caused a decrease in the dry weight of shoots and roots compared to control plants. Many researchers have explained that Cd inhibits the biochemical and physiological processes of plants by disrupting their metabolism, which cause growth inhibition of plants such as bean (*Phaseolus vulgaris*) (Howladar, 2014), cucumber (*Cucumis sativus*) (Nowak et al., 2014), tobacco (*Nicotiana tabacum*) (Iannone et al., 2015), and wheat (Çatav et al., 2020). These results suggest that Cd has a significant negative impact on the growth parameters of wheat seedlings and the degree of growth inhibition varies depending on exogenous proline concentration. In this study, the reason for the decrease in biomass in plants may be the change in the intake and distribution of essential nutrients (Eker et al., 2013). However, one of the negative effects of Cd on plant growth may be due to photosynthetic electron transport chain inhibition (Chen et al., 2011).

In addition, the growth of wheat under Cd stress increased by the addition of 1 mM proline. Similar to this study, it was stated that proline has a positive effect on growth in many plants (Hayat et al., 2013; Rasheed et al., 2014). Exogenous proline may have a protective effect on growth due to improvement in mineral nutrition (Dawood et al., 2014).

Accumulation of Cd and macronutrient contents of treated wheats

The transfers of Cd from the environment to plants pose a potential health risks because they are used for human consumption. Comparing measured Cd, Na, K, Ca, and Mg accumulations at five treatments between root and shoot tissues, Cd accumulation differences in root and shoot tissues occurred between entries for the five treatments analyzed (Figure 2).

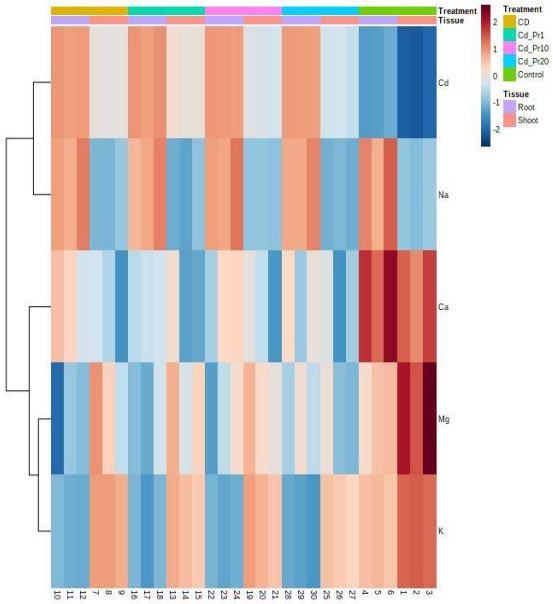

Figure 2. Heatmap analysis of the relationships between exogenously added proline and macronutrient content in tissue.

Results describing Cd accumulation in wheat seedlings showed that the increment of exogenous proline treatments (except Cd+Pr1) in the water resulted in a decrease of Cd content in roots and shoots. Additionally, Figure 3 showed that Cd+Pr20 supply reduced Cd accumulation in wheat. However, compared with the shoot, Cd accumulation was significantly higher in the root *(P<0.001)* in every treatment. Cd accumulations in roots was not significantly different between CD and all proline treatments whereas compared to control all treatments showed significant differences (*P<0.001*). The highest level of Cd was found in Cd+Pr1 treated wheat seedlings (6250.2 ppm DW in roots and 1054.6 ppm DW in shoots). These results suggested that concentrations of Cd in shoots could be different in all treatments In addition, the study results showed that approximately a 90-fold difference in Cd accumulation was found in roots and shoots between the lowest (control) and the highest treatments.

Table 3. Effects of different concentrations of proline on growth parameters of wheat seedling

Growth parameter	Treatment				
	Control	Cd	Cd+P1	Cd+P10	Cd+P20
Root length (mm)	92 ± 6^{a}	67 ± 3^{b}	70 ± 4^{b}	68 ± 4^{b}	63 ± 3^{b}
Shoot length (mm)	149 ± 4^{a}	108 ± 2^{b}	111 ± 2^{b}	107 ± 3^{b}	105 ± 2^{b}
Total seedling length (mm)	242 ± 9^{a}	175 ± 2^{b}	181 ± 6^{b}	175 ± 6^{b}	169 ± 4^{b}
Root/shoot ratio (length)	0.62 ± 0.04^{a}	0.63 ± 0.03^{a}	0.64 ± 0.03^{a}	0.66 ± 0.03^{a}	0.61 ± 0.02^{a}
Root dry weight (mg)	5.11 ± 0.12^{a}	3.85 ± 0.13^{d}	4.65 ± 0.15^{b}	4.40 ± 0.21^{bc}	4.22 ± 0.08^{c}
Shoot dry weight (mg)	12.17 ± 0.35^{a}	9.52 ± 0.39^{c}	10.57 ± 0.31^{b}	10.18 ± 0.28^{bc}	10.20 ± 0.22^{b}
Total seedling dry weight (mg)	17.27 ± 0.40^{a}	13.37 ± 0.50^{d}	15.21 ± 0.40^{b}	14.58 ± 0.33^{bc}	14.42 ± 0.22^{c}
Root/shoot ratio (dry weight)	0.43 ± 0.01^{a}	0.42 ± 0.01^{a}	0.45 ± 0.01^{a}	0.44 ± 0.02^{a}	0.42 ± 0.01^{a}

Values represent the means of 3 replications per treatment ± SD. Different letters indicate significant differences between treatments. Values with different superscript letters in the same row are significantly different from each other (P < 0.05, Tukey test).

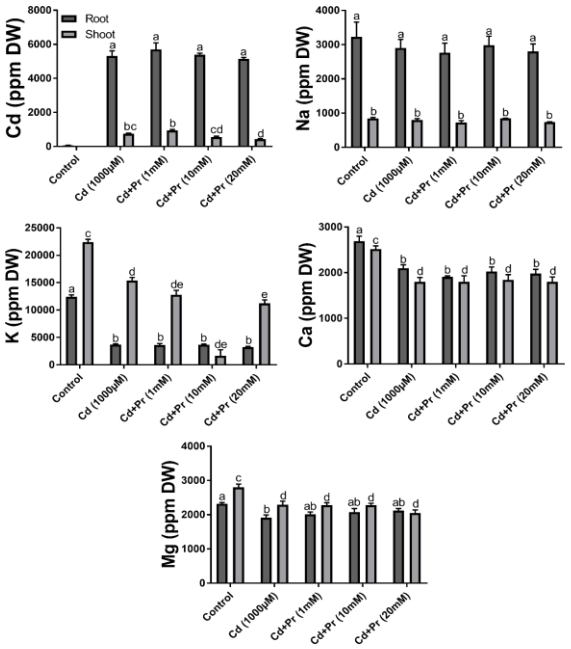

Figure 3. Cadmium (Cd), sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) contents of wheat seedlings subjected to different treatments. Results are presented as mean ± SE (n = 4) ($P < 0.001$; Tukey's HSD test).

At Cd treatment, the contents of Ca, K, Mg, and Na decreased in roots by 22.1, 70, 17.7, and 10.1%, and in shoots by 29.6, 32.2, 19.1, and 5.3%, respectively. The Mg contents of shoots are not significantly (*P<0.001*) changed under the proline applications in different concentrations, but Mg decreased in all treated wheat seedlings as compared to control groups. In addition, K content in both root and shoot has decreased in all exogenous applications. Figure 3 shows the effect of exogenous proline treatments on mineral uptake in wheat seedlings grown under Cd stress. No significant statistical differences occurred between all proline applications and Cd treatment in terms of Ca and Mg content in the roots.

In addition, no statistical difference (*P<0.001*) was found for Na in roots and shoots in all treatment groups. In Cd+Pr10 treatment compared to Cd treatments, a slight non-significant increase was observed in roots by 8 and 3.7% and in shoots only 5.7% for Na. Pietrini et al. (2005) reported that the presence of Cd ions may affect the uptake and transport of nutrients by plants. Gonçalves et al. (2009) reported that the uptake of Fe, Mn, Cu, and Zn is restricted in Cd-stressed potatoes (*Solanum tuberosum*). In a similar result, Ali et al. (2014) stated that Cd inhibits the absorption of macro elements such as Mg, Ca, and K in oilseed rape (*Brassica napus*). Accumulation of Cd in wheat is closely related to factors such as environmental conditions and the degree of tolerance of species.

Conclusion

In order to deduce the ability of wheat to grow under the Cd-contaminated and different concentrations exogenous proline in water, (i) the effect of different concentrations of exogenous proline on Cd accumulation in root and shoot, (ii) the impact of exogenous proline on growth, and (iii) macronutrient contents were investigated in this study.

According to the obtained results, the roots showed more Cd accumulation than the shoots in the wheat treated with Cd and exogenous proline in its water. The reason for the high Cd accumulation in the roots of plants could be explained by the decrease in the level of free Cd ions through a rapid metal-binding chelate or protein production (Hossain et al., 2012). The roots of wheat appear to be transported into aerial parts and act as an effective barrier against Cd accumulating. Xu et al. (2009) reported that proline application reduced the ROS and protected the callus plasma membrane from Cd stress. Thus, regeneration occurs in *Solanum nigrum* shoots. Sharma et al. (1998) found that exogenous proline protects nitrate reductase *in vitro* against inhibition by Cd. Metal-proline complex formation provides this protection. The reduced accumulation of Cd in wheat treated with exogenous proline may be due to the inhibitory effect of proline on Cd translocation and uptake. Proline limits the absorption of toxic metals in different species described above and also in the wheat used in this study.

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RESEARCH PAPER

In vitro androgenesis in pepper (*Capsicum annuum* L.) and the affecting factors on success: II. Carbohydrate source and antioxidants

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Abstract

Microspore cells develop into male gametophytes, which are released as pollen. Under certain stress conditions, the developmental pathway of microspores can be transformed into an embryo instead of pollen with haploid technology. In this experiment, 2 pepper breeding lines (G-1 and G-2) and 4 nutrient media formed with Murashige & Skoog (1962-MS) + 30 g L^{-1} sucrose or maltose and with or without vitamins as antioxidants. 0.05 mg L^{-1} biotin (vitamin B7) and 0.5 mg L^{-1} ascorbic acid (vitamin C) were studied as antioxidants. The anthers were pretreated for 2 days at 35 ºC in dark, then they were incubated in a climate chamber at 25 ºC and 16/8 hours photoperiodic conditions. The highest embryos rate, and development of embryos into the plantlet has been obtained from the medium containing maltose, and antioxidants. Although there was no significant differences between genotypes in medium- I (MS + sucrose), medium-II (MS + sucrose and antioxidants) and medium-III (MS + maltose) in terms of embryogenic response, a significant difference was determined between genotypes in medium-IV (MS + maltose and antioxidants). The number of embryos obtained from the G-1 in medium-IV has increased 2.5, 6.4, and 4.5 times, compared to the medium-I, medium-II, and medium-III respectively.

Introduction

Doubled haploidy (DH) system is a widely used tool in plant breeding, genetic research, and biotechnology today. The developmental pathway of a gamete cell can be changed and directed towards sporophytic development instead of mature pollen from microspore with double haploid technology. Accordingly, a gamete cell can form a haploid embryo alone, under certain stress conditions (Segui-Simarro & Nuez, 2008; Shariatpanahi et al., 2006; Perez-Perez et al., 2019; Testillano, 2018, 2019; Cengiz & Korkut, 2020). Stress pretreatments applied to anthers and microspores to inducing the sporophytic pathway are inhibit the gametophytic pathway and increase embryogenic potential (Sanchez et al., 2020). Microspores-induced embryo formation is called androgenesis.

In pepper anther culture, 35°C temperature pretreatment of anthers is the most commonly used stress condition. However, the levels of intracellular reactive oxygen species increase in microspores exposed to high temperature stress conditions and negatively affect the viability of microspores (Zur et al., 2009; Varnier et al., 2009; Gill & Tuteja, 2010).

Antioxidants reduce the oxidative stress in the plant and ensure the viability of microspores. Vitamin C (ascorbic acid) and vitamin B7 (biotin) are excellent free radical scavengers. They can provide tolerance to osmotic and oxidative stress in plants (Roje, 2007).

In *Capsicum annuum* L.; although different protocols have been employed to increase the productivity of anther culture during the 48 years since the first successful androgenic embryogenesis reported by Wang et al. (1973), George & Narayanaswamy (1973), and Kuo et al. (1973), both embryo productivity and plantlet regeneration rate still have not reached the desired level.

Developing a successful protocol for producing DH plants in each plant species and variety is difficult and may take years. It is known that various factors such as donor plant genotype and growth conditions, culture methods, composition and hormonal composition of the nutrient medium, stress pretreatment that induces embryogenesis, and flower bud development period affect embryo productivity. Although protocols for the production of DHs exist for many species, conditions suitable for one species or one genotype may not work for another (Segui-Simarro & Neuz, 2008; Irikova et al., 2011; Çömlekçioğlu & Ellialtıoğlu, 2018). Therefore, these factors need to be studied in detail in order to precisely determine the combination that works for the donor plant genotype studied.

In order to encourage microspores to embryogenic development even in recalcitrant species, new targets need to be determined and new strategies should be devised. Therefore, in this study, the effects of two factors on the yield of haploid embryo, the carbon source (sucrose and maltose) used in the nutrient medium and the use of antioxidants (ascorbic acid and biotin), were investigated in two capia type pepper breeding lines.

Materials and Methods

This study was carried out in the greenhouse and tissue culture laboratories of United Genetics Vegetable Seeds Company (Mustafakemalpaşa, Bursa, Turkey). Two capia type pepper (*Capsicum annuum* L.) breeding lines of the company coded as G-1 and G-2 were used as the donor plant. Donor plants were grown in an unheated greenhouse. Donor plant seedlings were planted in the unheated greenhouse of United Genetics Turkey Vegetable Seeds Company in Mustafakemalpaşa/Bursa location on April 13, 2020. Flower bud harvesting for anther culture started on 20 May and continued until June 15. Table 1 shows the certain climatic variables of greenhouse for the experimental year for the months which experiments were conducted (from donor plants planting to the end of flower buds harvested). According to their morphological development, the buds in the developmental stage where the corolla, and calyx are the same height or the corolla height is slightly above the calyx height were collected and brought to the laboratory. Anthers at this developmental stage mostly have late uninucleate, and middle uninucleate microspores or are known to contain microspores in the young binucleate stage (Çömlekçioğlu & Ellialtıoğlu, 2018).

Table 1. Temperature and relative humidity conditions of greenhouse during the experiments

Year (2020)	Minimum temperature (°C)	Average air temperature (°C)	Max. air temperature (°C)	Average relative humidity
April	13	24.0	35	48.6
May	16	26.5	37	47.9
June	17	27.0	38	54.2

For surface sterilization, flower buds were first rinsed with water, then with 70% ethanol for 10-15 seconds and kept in 10% commercial bleach (containing 5% sodium hypochlorite) and a drop of Tween-20 for 10 minutes. It was washed 3 times with sterile distilled water. The buds were carefully cut and isolated in a laminar flow cabinet on sterile paper.

In the experiment, four MS (Murashige & Skoog, 1962) culture media, supplemented with sucrose or maltose, and with or without 5 mg L^{-1} ascorbic acid, 0.05 mg L^{-1} biotin combination (as antioxidants) were used. In our previous study, 15 g L^{-1}, 30 g L^{-1}, and 60 g L^{-1} maltose were tested and the best result was obtained from 30 g L^{-1}. Therefore, 30 g L^{-1} maltose was studied in this study (Bat et al., 2020). All chemicals used in this experiment were Duchefa Biochemie brand. The culture media used are:

Medium-I (M-I); MS + 4 mg L^{-1} naphthalene acetic acid (NAA), 0.5 mg L^{-1} benzyl amino purine (BAP), 0.25% activated charcoal (AC) 15 mg L^{-1} silver nitrate ($AgNO_3$), 7 g L^{-1} agar, 30 g L^{-1} sucrose,

Medium-II (M-II); MS + 4 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, 0.25% AC, 15 mg L^{-1} $AgNO_3$, 30 g L^{-1} sucrose, 7 g L^{-1} agar, 0.5 mg L^{-1} ascorbic acid, 0.05 mg L^{-1} biotin,

Medium-III (M-I); MS + 4 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, 0.25% AC, 15 mg L^{-1} $AgNO_3$, 7 g L^{-1} agar, 30 g L^{-1} maltose,

Medium-IV (M-IV); MS + 4 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, 0.25% AC, 15 mg L^{-1} $AgNO_3$, 7 g L^{-1} agar, 30 g L^{-1} maltose, 0.5 mg L^{-1} ascorbic acid, 0.05 mg L^{-1} biotin.

Anthers planted on nutrient media were exposed to heat pre-treatment at 35°C under continuous darkness for the first 2 days. It was then incubated at 25°C under photoperiod conditions for 16/8 hours.

Statistical Analysis

The experiment was carried out in a randomized block design with 3 replications. At each replication, 100 anthers were cultured in 10 Petri dishes containing 10 anthers each. The data were subjected to analysis of variance (ANOVA) using Tarist (Açıkgöz et al., 2004). Mean separation was performed by Fisher's Least Significance Difference (LSD) ($p<0.01$).

Table 2. Summary of ANOVA for total embryos obtained, embryo rate and regenerated plantlets

Source of variation	DF	Total embryos		Embryo rate (embryo per 100 anther)		Number of regenerated plantlets	
		Mean square	F-value	Mean square	F-value	Mean square	F-value
Genotype (G)	1	70.04	175.63**	81.29	154.67**	4.10	21.18**
Media (M)	3	160.82	403.25**	207.04	393.93**	30.23	156.31**
GXM	3	43.60	109.32**	30.01	57.09**	19.63	101.52**
Error	14	0.39		0.53		0.19	
Total	23	30.03		34.86		7.45	

*Significant at alfa level 5%; ** Significant at alfa level 1%; DF= Degrees of freedom

Results and Discussion

This study was conducted to examine the androgenesis response of pepper to the carbohydrate source (sucrose or maltose) and 'biotin + ascorbic acid' supplementation as antioxidants in the nutrient medium. In this study, 4 different nutrient media were tested. Variance analysis results are presented in Table 2. Approximately 2 months (58 days) after the anthers were cultured, the first embryos were seen and embryo formation continued for almost 5 months.

The number of embryos obtained showed significant differences according to genotype, nutrient medium, and 'genotype × medium' interaction. Both genotypes produced a higher number of embryos in M-I and M-IV compared to the M-II, and M-III. While G-I produced the highest number of embryos in M-IV, gave a similar number of embryos in M-II and M-III. G-2 produced a higher number of embryos in M-I and M-IV compared to the M-II, and M-III. In the M-IV, the difference between the 2 genotypes was found to be very significant (Table 3). In this experiment, it was determined that the genotype effect was more important than the nutrient medium.

Genotype × medium interaction was found important in terms of androgenesis rate (embryo obtained per 100 anthers). The highest rate of the embryo was obtained from G-1 in M-IV. Both genotypes had the lowest values in M-II containing sucrose, and ascorbic acid, and biotin. It has been determined that adjusting the most suitable nutrient medium according to the genotype is important in androgenesis.

Figure 1 shows a few microspore-derived embryos and fully developed green plantlets obtained from this experiment. While some of the embryos obtained from the culture develop into a complete plantlet, some embryos cannot form green plants. Accordingly, it was determined that the number of plantlets developed from the obtained embryos differed significantly in terms of genotypes, media, and their interactions. The lowest plantlet growth was determined in the M-II and the highest in M-IV, parallel to the number of the embryos obtained (Table 3).

The results showed that the interaction of ascorbic acid, and biotin combination with different carbohydrate sources is important. While determining the negative effects of ascorbic acid and biotin combination on embryogenesis in sucrose contained medium, it has been observed that ascorbic acid and biotin combination has a significant positive effect in maltose content medium. The type of sugar used in the nutrient medium had a significant effect on androgenesis, and maltose had a positive effect on inducing haploid embryo formation. The combination of 30 g L^{-1} maltose, 0.5 mg L^{-1} ascorbic acid, and 0.05 mg L^{-1} biotin was the best culture medium for induction of high-frequency embryogenesis (26.1% for G-1 and 16.4% for G-2). Both genotypes gave similar results in terms of embryogenic response in M-I, M-II, and M-III, whereas a significant difference was detected between genotypes in medium-IV. The number of embryos obtained from G-I increased 2.5, 6.4, and 4.5 times, compared to the M-I, M-II, and M-III respectively. The effect of the culture medium on the success of anther culture in interaction with the genotype was clearly demonstrated in the study.

Cheng et al. (2020) reported that stress pretreatments to anthers are necessary to induce embryo development from microspores, and heat stress applications in peppers are indispensable since plant regeneration cannot be achieved without heat pretreatment. Stress applications used to reprogram microspores for embryogenesis often cause cell death.

Table 3. Total number of embryos, embryo ratio and number of plantlets obtained according to nutrient media and genotypes

Medium	Number of embryos			Embryo rate (Embryo no/100 anthers)*			Number of the plantlets		
	G-1	G-2	Mean	G-1	G-2	Mean	G-1	G-2	Mean
M-I	7.7 b	8. 3 a	8.0 b	16.1 b	16.8 a	16.4 b	3.0 b	4.3 a	3.7 b
M-II	3.0 c	1.0 c	2.0 d	9.9 d	5.7 d	7.9 d	1.0 c	1.0 c	1.0 c
M-III	4.3 c	3.3 b	3.8 c	12.0 c	10.5 c	11.2 c	1.3 c	1.7 c	1.5 c
M-IV	19.3 a	8.0 a	13.7 a	26.1 a	16.4 a	21.3 a	9.3 a	3.0 b	6.2 a
Mean	8.5 a	5.2 b		16.0 a	12.4 b		3.7 a	2.8 b	
LSD (%)	G: 0.8, M: 1.1, G x M: 1.6			G: 0.9, M: 1.3, G x M : 1.8			G: 0.6, M: 0.8, G x M: 1.1		

G: Genotype, M: medium; GxM: GxM Interaction. Means followed by the same letter in column do not differ according to least significance difference (LSD) test (P≤ 0.01). *Arcsine transformation was performed for analysis of variance

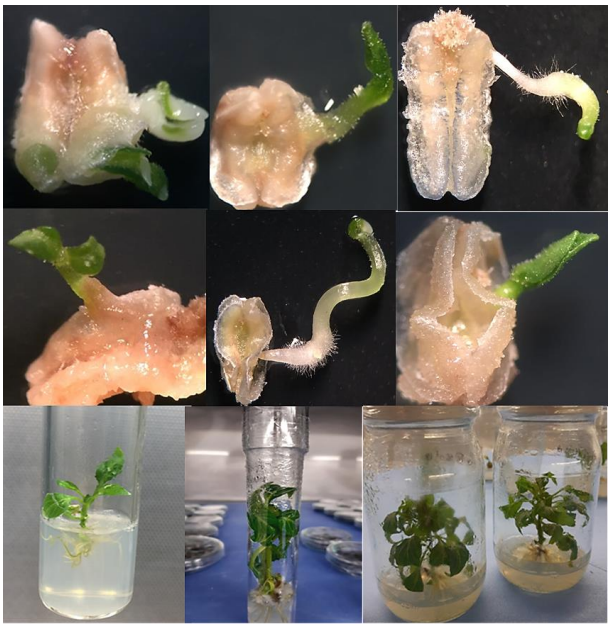

Figure 1. Some of microspore-derived embryos and plantlets obtained from the cultures.

It is the main factor that greatly reduces the efficiency of the method in transition to embryogenesis (Testillano, 2018; Rodriguez-Serrano et al., 2012). It limits the use of these methods in many plants because of low totipotens, and embryogenesis, thus appropriate stress conditions must be applied. Stress applied for the induction of embryogenesis causes an increase in reactive oxygen species (ROS) in microspore cells, and death (Zur et al., 2009; Rodriguez-Serrano et al., 2012). In order to prevent oxidative damage in the cell and to control cell death, ROS cleaning applications are of great importance. ROS scavenging roles of vitamins in plant cells have been reported previously (Becker et al., 2014; Zur et al., 2009; El-Sharabasy, 2019).

It has been reported that the vitamins in the culture medium increase the microspore viability and thus the embryogenesis response improves (Al-Khayri, 2001; Roje, 2007; Habibi et al., 2009; Hoseini et al., 2013; Zeng et al., 2015; Ozsan & Onus, 2017). It has been reported that it is important to increase the tolerance of microspore cells against oxidative stress caused by the stress pretreatments applied to anthers. Due to anti-oxidant properties of certain vitamins in the nutrient medium is a factor that increases the androgenic embryo yield (Demirkaya & Comlekcioglu, 2021). Navarro-Alvarez et al. (2006) reported that carbohydrates are critical components in anther culture medium for successful somatic embryo initiation, and plant regeneration. He reported that galactose and mannose did not support embryo formation, while glucose gave more positive results than sucrose and fructose. However, the highest rate of embryos in three wheat genotypes was obtained from the medium containing maltose.

In pepper anther culture, sucrose is the most commonly used carbohydrate source in culture media (Taskın et al., 2011; Çömlekçioğlu & Ellialtıoğlu, 2018). However, there are studies reporting that maltose as a carbohydrate source is more effective for androgenesis compared to sucrose. Last et al. (1990) reported that when maltose is used instead of sucrose in wheat anther culture, a three to four-fold increase in embryo yield was observed although it varied according to genotypes. Trejo-Tapia et al. (2002) found that maltose is the most effective carbohydrate in callus induction. Bat et al. (2020) compared 15 g L^{-1}, 30 g L^{-1}, and 60 g L^{-1} maltose doses with 30 g L^{-1} sucrose in a study with 3 pepper genotypes. While emphasizing the importance of the genotype effect in the androgenetic response, the study results also showed that a significantly higher embryo formation was achieved in a medium containing 15 g L^{-1} and 30 g L^{-1} maltose compared to a medium containing 30 g L^{-1} sucrose.

In a small number of trials using maltose (Irikova et al., 2011), maltose was generally used alone and different carbohydrate sources were not compared in the studied genotypes. Geboloğlu et al. (2017) reported that the use of honey instead of sucrose in eggplant anther culture has a positive effect on obtaining embryos, with the highest number of embryo formation being 18.35% and 20.95% from honey and sucrose treatment, respectively.

Although pepper is one of the most intensively studied plants on androgenesis, DH production in this species is still not at the desired level. The relationship between androgenetic response and nutrient medium composition in peppers has been widely accepted. However, although it is known that carbohydrate sources and doses in the nutrient medium affect embryogenesis, studies have focused on growth regulators and the relationship of their doses with the embryogenic potential of microspores rather than determining the most appropriate carbohydrate source, and doses.

It has been determined that the applications are very promising in terms of increasing the yield of haploid embryos with the anther culture method, as well as on the rate of obtaining plantlets from the formed embryos. It is thought that different carbohydrate sources and doses should be studied in also other recalcitrant species to increase the efficiency of haploid embryogenesis, and plantlet regeneration. While emphasizing the importance of the genotype effect once again in the study, it was determined that different factors should be studied in detail in pepper anther culture. Genotype effect in anther culture response is an important factor limiting the application of this technology in plant breeding. This requires the experimental development of culture media, and protocols for different genotypes. Culture protocols that can be successfully applied in different genotypes need to be developed, and optimized.

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Author Contributions

Conceptualization, writing-review and editing: NC, SSE; Methodology: FNA, MAY, ED; Data curation, Formal analysis: ED.

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