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SWEET PEPPER SEED GERMINATION AND SEEDLING GROWTH AFTER TREATMENT WITH SLUG MUCUS

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The development of new methods of crop production is the most promising field of research in agriculture. There are numerous chemical techniques of seed treatment before sowing; however, mollusc mucus has not been considered as a natural alternative of seed treatment yet. In this study, we treated sweet pepper seeds with Spanish slug mucus before sowing. Seed germination success, germination time, as well as the performance of seedling growth were compared between experimental and control groups. The main result showed that seeds treated with slug mucus required significantly fewer days to germinate than seeds without treatment. Although treated seeds exhibited faster germination, seedlings showed slower growth than in the control group. No significant difference of seed germination success between the control and experimental group was found.

Keywords: *slime,* Arion vulgaris, Capsicum annuum*, sprouts germination.*

INTRODUCTION

The increasing demand for food and the limiting area of farmlands encourage scientists to find new effective methods of crop production (Ramankutty *et al*., 2018). These methods should correspond to the requirements of food safety, as well as minimise the destructive effects on the environment (Valin *et al*., 2014). Synthetic chemical treatments have proved their efficiency in increasing the productivity of crops; however, they have potentially harmful side effects on the environment (Nicolopoulou-Stamati *et al*., 2016). Therefore, one of the most promising field of research is the development of sustainable, or environmentally friendly, methods of crop production (Bale *et al*., 2008). Biophysical, biochemical and biological seed treatments aim to improve tolerance and sprouting, manage pests and diseases, as well as to activate the growing process (Sharma *et al.*, 2015; Procházka *et al.*, 2016; Ishizuka *et al.*, 2020; Romero-Galindo *et al*., 2021).

In nature, molluscs secrete mucus to defend against predators, lubricate surfaces during locomotion, prevent loss of moisture, attach on the vertical substratum and form the epiphragm, as well as cover eggs during reproduction (Barker, 2001; Newar and Ghatak, 2015; Harti *et al*., 2016). Humans have found the application of mucus in several industries. For example, cosmetic companies offer products with various beneficial effects on the skin such as healing, anti-ageing, acne-fighting, and regeneration. Mucus may also find application in medicine, as it showed potential use as an alternative source of antibacterial agents in the control of infections caused by bacteria (Etim *et al*., 2016). The adhesive properties of mucus may be used in the development of glue or gels with unusual properties (Pawlicki *et al*., 2004). However, the potential application of mollusc mucus in an agricultural field has not yet been considered.

Snail mucus contains various elements such as carbon, oxygen, nitrogen, potassium, chloride, calcium, sodium, magnesium, phosphorus, and sulphur (Greistorfer *et al*., 2017), as well as adhesive (Pawlicki *et al*., 2004) and protective proteins (Otsuka-Fuchino *et al*., 1992; Adikwu and Alozie, 2007; Pitt *et al*., 2015; Cilia and Fratini, 2018) that act against bacteria, fungi and viruses (Trapella *et al*., 2018). The properties of mollusc mucus mentioned above seem to

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suggest potential application as a pre-sowing seed treatment.

In this study, we selected Spanish slug *Arion vulgaris* Moquin-Tandon, 1855 (Gastropoda: Pulmonata: Arionidae) for mucus sampling due to the protective properties of its slime (Li *et al.*, 2017; Greistorfer *et al.*, 2020). Moreover, the Spanish slug is a highly invasive slug species and causes negative economic and ecological impact in many European countries (Zemanova *et al.*, 2016). One of the most effective and environmentally-friendly methods of how to deal with this species is by physical removal of individuals by hand. This means that collected slugs and their mucus remain free from molluscicides and other chemicals. This could be a rational approach to collect *A. vulgaris* mucus before utilisation, thereby contributing in sustainable waste management and obtaining material for biological seed treatment. We hypothesised that mollusc mucus has a positive effect on seed germination and/or seedling growth. We expected that mucus would show disinfestation properties by destruction of surface-borne organisms that have contaminated the seed surface but not infected the seed, and that the mucus can protect young seedlings of sweet pepper (*Capsicum annuum* L. cultivar 'California Wonder') from organisms in the soil that might otherwise cause decay of seeds before germination. Although, the pepper is a widely used crop species in ecological, horticultural and genetic studies (Ben-Chaim and Paran, 2000; Díaz-Pérez, 2013; Omezine and Teixeira da Silva, 2016), pepper seeds are still poorly studied (Silva *et al.*, 2013).

The aim of this study was to test whether the ratio of germinated vs. dead seeds, sprouting time, as well as sprout length and weight, significantly differed between a control and experimental group (treated with *A. vulgaris* mucus) and to determine if there is potential use of mollusc mucus as a pre-sowing seed treatment.

MATERIALS AND METHODS

Mucus sampling. About 500 Spanish slugs were collected from natural populations in private gardens in Jēkabpils, southeastern Latvia, in July 2020. They were transported to the Study and Research Centre "Ilgas", Daugavpils University, and maintained in MLR-352H environmental test chambers (Panasonic Healthcare Co., Tokyo, Japan) at 17 ± 2 ^{o}C. Slugs were kept in plastic boxes (30 cm wide × 40 cm long × 34 cm high), covered inside with moist paper tissues to be convinced that slug mucus will not contain any residuals of soil. To exclude the presence of food in the mucus samples, slugs were not fed for one day before mucus collection. Ventilation in the boxes was provided by making holes in their lids. Slugs with visible abnormalities were excluded from the experiment. The care and use of laboratory slugs were performed according to the guide of the National Research Council (1996) and was approved by the Ethics Committee of the Daugavpils University. All manipulations complied with current laws in Latvia. All experimental trials were performed in July and August 2020.

Before mucus collection, the slugs were sprayed with ozone-infused water to remove bacteria and fungi. The slugs were treated with warm steam to make them release mucus. They were placed in metal sieves above the container with steamy water and were shaken moderately for about five minutes. Then mucus was collected manually using a sterile metal spatula. This method was used to prevent the slugs from suffering and to allow extraction of mucus from the same slugs more than once. The collected mucus was placed in sterile glass vials and stored in a refrigerator at 4 ± 2 ^{o}C for further use.

Experimental design. In total 252 seeds of sweet pepper (*Capsicum annuum* L. cultivar 'California Wonder') were sown in three repetitions. Each repetition was divided into two groups: control group (total weight = 0.786 g and mean weight of seeds = 0.006 g) and experimental group (total weight = 0.782 g and mean weight of seeds = 0.006 g). Each seed was weighed with an ADB 100-4 analytical balance (KERN & SOHN GmbH, Balingen, Germany) to the nearest 0.01 g and placed in an individual plastic Eppendorf tube (volume 1.5 ml). Each individual seed of the experimental group was temporally (for approximately three seconds) dipped in slug mucus and dried at room temperature for 24 h. The seeds of the control group were left in the Eppendorf tubes. On the next day, seeds from both groups were sown in individual black plastic containers (60 mm long × 60 mm wide × 70 mm high), filled with bio-humus based soil mixture (earthworm compost, cattle manure, milled peat, limestone meal) without any synthetic fertiliser. The seeds were sown at a depth of 10 mm. The individual containers were kept at a distance of 5 cm one from the other to provide enough space for each growing seedling. The soil was moistened after sowing and then every two days. Germination was recorded daily and was considered successful when both cotyledons emerged completely, whereas a seed was considered to be dead when not germinated at all or cotyledons did not emerge completely. Germination time was measured from sowing day till the emergence of both cotyledons. Twenty-one days after the appearance of cotyledons, the length of each seedling was measured from the surface of the soil till the top of the plant. At the end of all measurements, the total dry weight of each seedling was determined. Seedlings were dried at 70 °C for three days in a ventilated oven.

Conditions in environmental chambers. The containers with seeds were placed in non-industrial environmental chambers made by local manufacturers. The experiments were conducted in a 16 h light and 8 h dark photoperiod, with waterproof Philips Ledinaire lamps, which provided illuminance of approximately 6000 lux. The temperature and relative humidity in the environmental chambers were chosen to be optimal for pepper seed germination, respectively, 26 ± 2 ^{o}C and 70–85 %. Additional ventilation was provided to guarantee sufficient air circulation in the chambers.

Statistical data analysis. A one-way MANCOVA was conducted to test for statistically significant differences of the

adjusted means of combined dependent variables (sprouting days, length and weight of sprout) between control and experimental groups after controlling for the effect of the covariate (seeds weight). A correlation matrix was calculated to examine multicollinearity between the dependent variables. The Shapiro–Wilk multivariate normality test was used to determine whether combined dependent variables were approximately normally distributed. The Levene's test was used to determine whether there were equal variances between the groups of the independent variable for each dependent variable. The Box's M test was conducted to examine the assumption of the equality of covariance matrices. To test the independence of the covariate and treatment effect, an ANOVA was run between the groups of each independent variable with the covariate as the dependent variable to determine independence (Field, 2013). The assumption for homogeneity of regression slopes was assessed by MANCOVA and including interaction terms between each independent variable and covariate (Stevens, 2009). The analysis of germination success was analysed by the test of two proportions where the independent variable was treatment with two categories: control and experimental group, and the dependent variable was germination with two categories: germinated and not germinated (died). Data validation and analysis were implemented in R version 4.0.0 (R Development Core Team, 2020) using the packages car, mvnormtest, heplots. effects, plyr and multcomp.

RESULTS

The covariate (seeds weight) was significantly related to the combined sprout length and weight $\Lambda = 0.954$, $F(1, 195) = 4.606$, $p = 0.011$, partial $^2 = 0.050$. The result of the one-way MANCOVA was significant $\Lambda = 0.752$, $F(1, 195) = 31.895$, $p < 0.001$, indicating a significant difference in combined dependent variables between control and experimental groups after controlling for the effect of seeds weight. According to Cohen (1988), the partial $^2 = 0.438$ indicates the large effect size of this difference. Post hoc analysis of the sprout length revealed that the covariate-adjusted mean of the control group (adjusted mean = 99.78 mm, standard deviation = 10.93 mm) was significantly larger than that of the experimental group (adjusted mean = 86.94 mm, standard deviation = 15.62 mm); the statistically significant difference was 12.83 mm, 95% CI [9.07–16.59]. Post hoc analysis of the sprout weight revealed that the covariate-adjusted mean of the control group (adjusted mean = 1.88 g, standard deviation = 0.50 g) was significantly greater than that of the experimental group (adjusted mean =1.42 g, standard deviation = 0.45 g), the statistically significant difference was 0.45 g, 95% CI [0.32–0.58]. Post hoc analysis of the sprouting days revealed that the covariate-adjusted mean of the control group (adjusted mean = 9.63 days, standard deviation = 1.40 days) was significantly greater than that of the experimental group (adjusted mean = 8.27 days, standard deviation = 0.87 days), the statistically significant difference was 1.36 days, 95% CI [1.04–1.68] (Fig. 1). The difference of seeds germination success between control and experimental group was not recorded ($p > 0.05$). In the control group, 96 seeds germinated, and 30 seeds died whereas, in the experimental group, 95 seeds germinated, and 31 seeds died.

DISCUSSION

Studies with peppers have reported positive or negative effects of different seed treatments on both two developmental processes: germination and seedling growth (Demir *et al.,* 2008; Samarah *et al.*, 2016). For example, Tombegavani *et al.* (2020) showed that sweet pepper seed treatment with plant hormones improved seed germination and growth traits of young plants. However, Hassen with colleagues (2014) found that increased levels of salt stress negatively affected all germination and seedling growth parameters in *C. annuum.* We also expected either positive or negative effect of mucus on seed germination and consistently on post-germination performance in our study, but the main results showed mixed effect — slug mucus faciliated faster

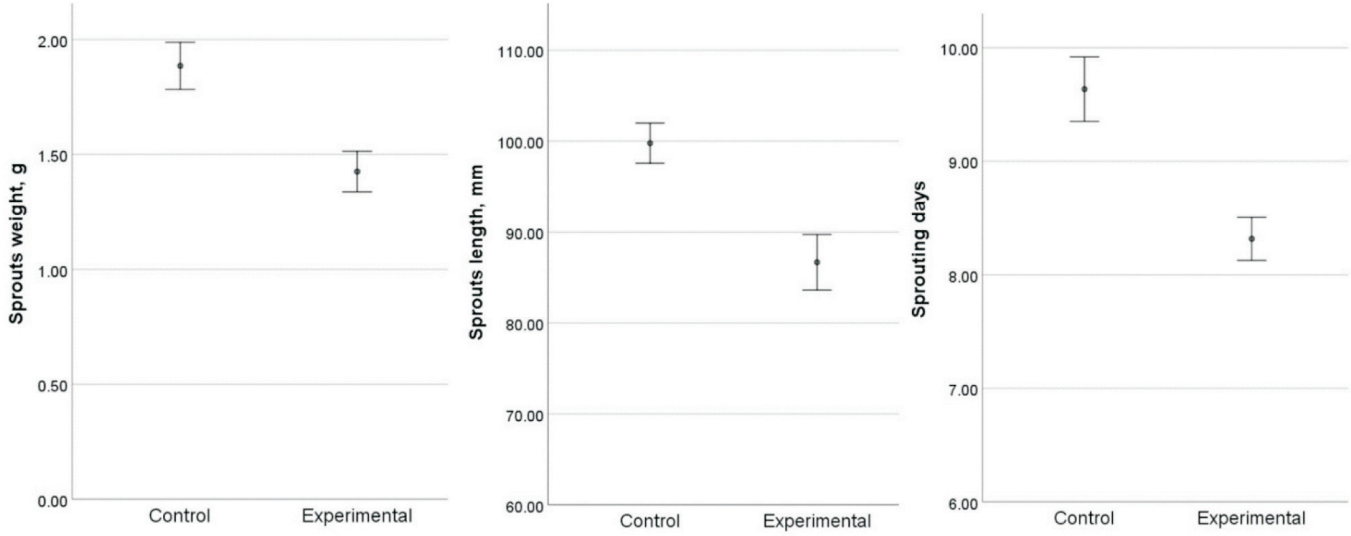


Fig. 1. The mean value and confidence interval of sprouting days, length and weight of sprouts for control and experimental groups.

seed germination, but further sprout growth was slower than in the control group. We observed that seeds treated with slug mucus require significantly fewer days to germinate than seeds without treatment. In other words, our expectations about the slug mucus effect on seed germination and sprout growth were not supported. Such results were also found in some other seed germination and seedling growth studies. Mohsenkhah *et al.* (2018) exposed seeds and seedlings of *C. annuum* to microwave radiation and found that the positive effect depended on the exposure time, which was significantly different for each development stage; seeds required 10 h of exposure for a positive effect from treatment (to reduce mean germination time), but for seedling growth this exposure time had a highly negative effect. Jorritsma-Wienk *et al.* (2006) showed that *Tragopogon pratensis* seed germination and seedling growth phases responded differently to the micro-environment. Hong *et al.* (2009) reported that the presence of polycyclic aromatic hydrocarbon contaminants stimulates germination and depresses seedling growth. Donohue *et al.* (2010) suggested that response to an environmental stressor may differ between germination and post-germination stage of plant, depending on the range of environmental conditions they experience. In future studies it is necessary to conduct detailed mucus chemical analysis, because some studies show the role of organic and inorganic compounds in seed germination and plant growth processes (Khan *et al.*, 2012; Amit and Abhinav, 2018; León-Morales *et al.*, 2019) and it is possible that such compounds are also present in mollusc mucus. Our results showed positive effect of mucus treatment on seed germination but negative effect on seedling growth. Therefore, the unknown factor responsible for such contradictory results should be identified and analysed. However, it is already clear that slug mucus has potential for further research not only in fundamental but also in applied agricultural studies. In this study, seed weight was significantly related to the sprout length and weight; however, this relationship had a small effect size (partial $\eta^2 = 0.050$) indicating low practical importance. Moreover, the difference between mean values and adjusted mean values indicated the unreasonable application of seeds weight as a covariate in further studies of mucus effect on seed germination and seedling growth. Further studies should test the following hypotheses: 1) does the mucus effect on seed germination and seedling growth differ between mollusc species; 2) can mollusc mucus act as priming agent that hydrates seeds to allow germination processes to take place, but not seedling growth; and 3) does the mucus effect on seed germination and seedling growth differ if collected from calm versus stressed molluscs.

Rehydration of seed seems to be the major factor of germination, as seeds store specific mRNAs and proteins that reactivate cells after infiltration (Rajjou *et al.*, 2012). Also, calcium helps to control the transition from dormancy to germination and has a nutritive role in seed germination (Roux, 1989). In mollusc mucus, water is the main component that swells mucus-forming proteins, whereas calcium plays a role in cross-linkage and viscosity of mucus (Deyrup-Olsen *et al.*, 1983; Deyrup-Olsen and Martin, 1987; Pawlicki *et al.*, 2004). Although in our experiment seeds were dipped in mucus for only a short time (not enough to provide water for germination), the established mucus layer around seed may act like a sponge that absorbs and stores water around the seed.

A. vulgaris start laying eggs in autumn and most eggs hatch before winter. Most *A. vulgaris* individuals overwinter in the juvenile stage. In the spring, overwintered juveniles proceed growing as soon as the temperature starts to rise and the first food appears (Kozlowski, 2007). We found that seeds treated with slug mucus germinated significantly faster than untreated ones. Perhaps, the mucus of adult slugs stimulates seed to germinate faster to provide juvenile stages with food as quickly as possible, because *A. vulgaris* has an annual life cycle. If mucus helps to absorb water and provides a stimulus for passing from dormancy to germination, then temperature is the only limiting factor of seeds germination. The temperature increase is related to the growth rate of plants as well as with the increasing size of *A. vulgaris* juveniles (Slotsbo *et al.*, 2013), which is beneficial for the animals because of food limitation during the spring. As slugs, especially young juveniles, feed mainly on young and delicate plant organs (Kozlowski, 2005), perhaps this is the reason why, in our study, seedlings had a smaller size than that of the control group. Nevertheless, our hypothesis on the effect of slug mucus on seed germination, as well as inhibition of seedling growth to facilitate slug juvenile survival and growth should be carefully tested. Therefore, study of slug mucus effect on seeds will be continued.

CONCLUSIONS

The findings of this study revealed the potential application of mollusc mucus in seed treatment before sowing, as well as showed the ecological role of mollusc mucus in seed germination. However, it is premature to consider the practical use of mollusc mucus in agriculture since we have received a dual effect on seeds — negative and positive. The results are also affected by the chemical composition of the mucus, which can vary greatly from species to species and depending on environmental conditions (Skingsley *et al.*, 2000; Ng *et al.*, 2013). We find it interesting that there is an indirect relationship between the life cycle of the slug and the effect of mucus on seeds, and thus we suggest firstly focusing on the ecological role of mucus and then investigating practical application in agriculture, to provide reliable recommendations in the future.

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SALDĀS PAPRIKAS SĒKLU DĪGTSPĒJA UN DĪGSTU AUGŠANA PĒC APSTRĀDES AR KAILGLIEMEŽU GĻOTĀM

Jaunu metožu izstrāde lauksaimniecības kultūru ražas uzlabošanai ir viens no perspektīvākajiem zinātnes virzieniem lauksaimniecībā. Eksistē vairākas metodes, ko izmanto sēklu apstrādei pirms sēšanas, tomēr gliemju gļotu izmantošana sēklu apstrādē kā dabiska alternatīva ķīmiskajām metodēm līdz šim nav pētīta. Šajā pētījumā saldās paprikas sēklas pirms sēšanas tika apstrādātas ar Spānijas kailgliemežu gļotām. Tika salīdzināta sēklu dīgtspēja, dīgšanas laiks un dīgstu augšanas parametri starp kontroles un eksperimentālo grupu. Pētījuma laikā tika konstatēts, ka sēklas, kuras tika apstrādātas ar kailgliemežu gļotām, izdīga būtiski ātrāk nekā ar gļotām neapstrādātās sēklas. Lai gan eksperimentālās grupas sēklas izdīga ātrāk, dīgstu turpmākā augšana notika lēnāk nekā kontroles grupā. Pētījuma gaitā netika konstatētas atšķirības sēklu dīgtspējā starp eksperimentālo un kontroles grupu.