

# *In vitro* germination and viability of pea pollen grains after application of organic nano-fertilizers

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## SUMMARY

The objective of this study was to evaluate the influence of two organic nanofertilizers, Lithovit and Nagro, on *in vitro* germination, pollen tube elongation and pollen grain viability of *Pisum sativum* L cv. Pleven 4. The effect of their application was high and exceeded data for the untreated control (44.2 and 47.23 % regarding pollen germination and pollen tube elongation, respectively), as well as the effect of the control organic algal fertilizer Biofa (17.5 and 27.9 %, respectively). Pollen grains were inoculated in four culture media. A medium containing 15% sucrose and 1% agar had the most stimulating impact on pea pollen grains. Pollen viability, evaluated by staining with 1% carmine, was within limits of 74.72–87.97%. The highest viability of pollen grains was demonstrated after the application of Nagro organic nano-fertilizer.

**Keywords:** Organic fertilizers; Pollen; Germination; Peas

## INTRODUCTION

Knowledge of the viability and capacity of pollen germination, aside from pollen tube growth, is crucial for investigation of the reproductive biology and genetic breeding of some plants, showing the direction and underlying controlled hybridization aimed at creating new hybrids and/or raising pollen viability (Dane et al. 2004; Salles et al., 2006). Low germination percentages and slow elongation of pollen tubes may influence seed formation. Studies of *in vitro* pollen germination and pollen tube growth are important for understanding fertilization and seed formation in flowering plants and are very useful for explaining any lack of plant fertility (Büyükkartal, 2003). Many factors are able to affect *in vitro* pollen germination: botanic species, cultivar,

plant nutritional state, culture medium, temperature, pollen sampling time, photoperiod, sampling method, application of fertilizers or pesticides to plants, pollen storage conditions, etc. (Stanley & Linskens, 1974; Neves et al., 1997). There are relatively few studies that have determined the effects of organic fertilizers (manures, biofertilizers based on microorganisms or plant extracts, etc.) on flowering characteristics, pollen germination in particular (Hassan et al. 2015). Furthermore, estimates are lacking on the influence of nano-fertilizers on pollen viability and *in vitro* pollen germination in different plant species. Nano-fertilizers are innovative products with some unique features, such as ultra-high absorption, increased yield, more intensive photosynthesis, but scant literature reports on the subject are available in scientific journals (Sekhon, 2014; Manjunatha et al., 2016).

In the laboratory, pollen viability can be determined quantitatively in media that stimulate pollen grain development or by using dyes, such as anilin blue, propionic carmine, acetic carmine, IKI (iodine + potassium iodide), Alexander's stain, etc. (Bolat & Pirlak, 1999; Wang et al. 2004). Dye tests, used as indicators of pollen viability, have the advantage of being a faster and easier method than experiments with *in vitro* pollen germination. However, different dye types may produce different results. Truly viable pollen can be quantified only by *in vitro* germination tests because cultivation conditions allow an adequate expression of the physiological capacity of pollen tube formation (Bolat & Pirlak, 1999).

The aim of this experiment was to test the effects of two organic nano-fertilizers (Lithovit and Nagro) on *in vitro* pollen grain germination, pollen tube growth, as well as pollen viability in pea (*Pisum sativum* L. cv. Pleven 4).

## MATERIAL AND METHODS

### *In vitro* pollen germination

For determining *in vitro* pollen germination, flowers of pea plants in anthesis were collected early in the morning. The pea plants (cv. Pleven 4) were previously treated (twice) at the growth stage 55 (BBCH-scale) with either of the two organic leaf nano-fertilizers: Lithovit (containing CaCO<sub>3</sub>, MgCO<sub>3</sub>, Fe) at a concentration of 0.2% or Nagro (containing the elements N, P, K, Mg, Zn, Fe, Cu, Mo, B, Ca, Se, etc.) at a concentration of 0.05%. The interval between two treatments was 10 days. The fertilizers were applied by a small-volume Matabi hand sprayer, and the solution volume was 20 l da<sup>-1</sup>. The control was watered with the same volume of distilled water. The organic leaf fertilizer Biofa (brown algae [*Ascorphyllum nodosum*] extract, extremely rich in macro- and microelements, alginic acid, natural plant hormones, PGR enzymes, etc.) was used at a concentration of 0.5% as the second control. Biofa had also been examined in our earlier field experiments. It showed a high effectiveness (on yield and nutritional value of forage crops), which was very comparable to the effects of conventional fertilizers.

Pollen grains of 10 flowers per variant were collected and then inoculated in Petri dishes (9 cm diameter) containing 40 ml of culture medium, using a brush for homogenous distribution of material. Since different media may affect germination results (Stanley & Linskens, 1974), four culture media were used in the present experiment: medium A - 15% sucrose; medium B

- 15% sucrose, 100 mg/L H<sub>3</sub>BO<sub>3</sub>, 300 mg/L Ca(NO<sub>3</sub>)<sub>2</sub>; medium C - medium A with additional 1% agar; medium D - medium B with additional 1% agar. The dishes were subdivided into quadrants, each one representing a replication with approximately 100-150 pollen grains, totaling 12 replications for each culture medium.

After inoculation, the dishes were kept at controlled temperature conditions (26 °C) for 24 hours (Nikolova et al., 2012) before reporting the germinated pollen grains and pollen tube length (stereomicroscope, magnification 10x). After 24 h, tube growth was stopped by adding 10% ethanol (Cresti & Tiezzi, 1992). Six microscopic areas (per quadrant) were counted randomly for evaluation of pollen germination and for measuring pollen tube length in each Petri dish. Pollen grains were assumed to have germinated when the pollen tube length was equal to or longer than the diameter of the pollen grain itself. The length of pollen tube was measured directly with an ocular micrometer fitted to the microscope eyepiece based on the micrometer scale (μm) (Sharafi et al., 2011). The experimental design for pollen germination / pollen tube length was double factorial: 4 organic treatments /Lithovit, Nagro, Biofa, control/ × 4 cultural media /A, B, C, D. The data in regard to pollen germination percentage were previously transformed to arcsin  $\sqrt{x(\%)/100}$ .

### Pollen viability

Pollen grains from the anthers of pea plants tested *in vitro* were excised and stained on glass slides, each with a drop of 1% carmine (Coser et al., 2012). They were covered with coverslips, and after a couple of minutes observed under the microscope (10 x lens). To determine the viability of pollen, three anthers per organic treatment variant were analyzed and 100 pollen grains/slide were counted. The percentage of pollen fertility was evaluated based on the proportion of stained pollen grains (viable) against unstained grains (non-viable). Pollen viability percentage was also transformed to arcsin  $\sqrt{x(\%)/100}$  prior to statistical analysis.

The obtained data were statistically processed using the software Statgraphics Plus for Windows Ver. 2.1 at LSD 0.05%.

## RESULTS AND DISCUSSION

The study revealed a positive effect of the application of organic leaf fertilizers on *in vitro* pollen germination and pollen tube elongation in pea plants (Table 1). The highest germination percentage, as well as the greatest pollen tube length, regardless of cultural medium,

were observed after applying the organic nano-fertilizer Nagro (45.04% and 588.58  $\mu\text{m}$  on average, respectively), followed by Lithovit (40.83% and 558.60  $\mu\text{m}$  on average, respectively). These values of the two parameters significantly exceeded those of the organic fertilizer Biofa (by 17.5 and 27.9% on average regarding pollen germination and pollen tube length, respectively) and the control (by 44.2 and 47.23%, respectively). Overall, the higher the germination percentage, the higher is the chance for fertilization (Salles et al., 2006). Consequently, it is important to find adequate organic products which have positive effect on pollen grain germination, which ultimately results in higher plant fertility. Hassan et al. (2015) also reported an increased pollen germination after using different organic fertilizers (poultry manure, sheep manure, a biofertilizer consisting of *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus circulans*) and their combinations. According to Bhangoo et al. (1988), such enhancement may be attributed to stimulating effects of the absorbed nutrients on the photosynthetic process, which certainly reflected positively on flowering characteristics, including pollen germination. The high effect of fertilizers is due to the nano dimensions of their particles, as well as the presence of boron in Nagro which is directly influencing the processes of flowering and pollination of forage crops (Pavlov & Kostov, 2001). Positive effects of a variety of nano-materials, mostly metal-based and carbon-based nano-materials, on growth and development of different crop plants have been revealed (Sekhon, 2014). Sheykhbaglou et al. (2010) reported an improvement in agronomic traits (pods, grain yield) of soybean after

using nano-iron oxide. However, not one report has been made on changes in pollen germination after using organic nano-fertilizers.

Under the conditions that existed in our present experiment, the variation in pollen tube growth in different media (A, B, C, D) was considerable. In the culture medium C, disregarding the organic fertilizer application, the mean pollen germination was highest (77.06%), as well as pollen tube length (936.49  $\mu\text{m}$ ), and it was followed by medium D (45.04% and 592.94  $\mu\text{m}$ , respectively). The lowest values were recorded in culture medium A, containing only sucrose. According to Gwata et al. (2003), differences in *in vitro* pollen grain germination resulted from a complex interaction between the morphology and physiology of pollen grains and components of the medium. For germination, a medium should contain some nutrients (e.g., calcium, magnesium sulfate, potassium nitrate or boric acid) (Soares et al., 2008). Agar added to such media provides stability, so that the growth of pollen tubes can be observed (Martin, 1972). In general, the medium for *in vitro* pollen germination varies depending on plant species and cultivar (Dane et al. 2004; Frazon et al., 2005). Still, little information is available on pollen tube growth in pea. Nikolova et al. (2012) found that an optimal medium for *P. sativum* contained agar, sucrose,  $\text{H}_3\text{BO}_3$  and  $\text{CaCl}_2$ . Our results are in accordance with previous studies (Warnock & Hagedorn, 1956) reporting that 15% sucrose and 1% agar made the most adequate medium.

Pollen staining tests are among the most reliable and most widely used pollen viability tests (Cresti & Tiezzi, 1992).

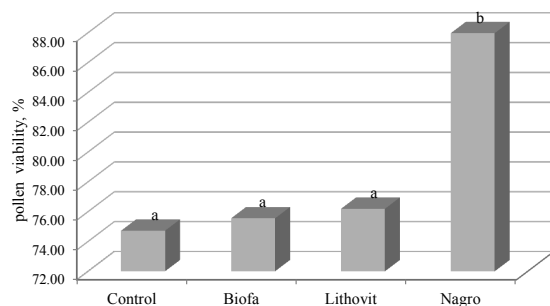
**Table 1.** Influence of organic fertilizer treatments and cultural media on *in vitro* pollen germination and pollen tube length of *Pisum sativum*

Organic Treatments	Cultural media			
	A*	B	C	D
	Pollen germination, %			
Control (C <sub>1</sub> )	4.05 a	11.60 b	59.11 h	44.33 f
Biofa (C <sub>2</sub> )	15.25 c	20.32 d	63.50 i	47.10 g
Lithovit	19.19 d	23.57 e	71.17 j	49.40 g
Nagro	19.59 d	25.39 e	77.06 k	58.12 h
	Pollen tube length, $\mu\text{m}$			
Control (C <sub>1</sub> )	109.72 a	226.25 c	708.33 i	514.07 g
Biofa (C <sub>2</sub> )	127.74 a	261.86 d	880.97 k	524.28 g
Lithovit	197.18 b	358.00 f	1093.45 m	585.76 h
Nagro	211.55 bc	331.92 e	1063.18 l	747.65 j

Means marked by the same letter did not differ statistically at 5% probability

\*medium A - 15% sucrose; medium B - 15% sucrose, 100 mg/L  $\text{H}_3\text{BO}_3$ , 300 mg/L  $\text{Ca}(\text{NO}_3)_2$ ; medium C - medium A with additional 1% agar, medium D - medium B with additional 1% agar

Fast estimation of pollen viability is of great value to plant breeders and geneticists in eliminating the time and space problems (Khosh-Khui et al., 1976). Pollen pea viability (cv. Pleven 4), evaluated by staining with 1% acetic carmine, was within limits of 74.72-87.97% (Figure 1). The highest, and statistically significant viability was demonstrated by pollen grains after the application of Nagro organic nano-fertilizer. Although pollen viability was higher after treatment with Lithovit and Biofa than in the control, the differences were not statistically significant. As a whole, the trends regarding pollen grain viability after different organic fertilizer applications corresponded to the trends regarding *in vitro* germination percentage and pollen elongation.



**Figure 1.** The influence of organic nano-fertilizers on pollen viability of *Pisum sativum*

In conclusion, the experiment revealed a positive effect of the application of organic nano-fertilizers Lithovit and Nagro on *in vitro* pollen germination and pollen tube elongation in *P. sativum*. The effect exceeded that of the organic algal fertilizer Biofa (by 17.5 and 27.9%, in regard to pollen germination and pollen tube length, respectively) and the control (by 44.2 and 47.23%, respectively). The obtained results enrich the current information about the activity of nano-fertilizers. In the future, more detailed research is needed to clarify the mechanism of action and the consequences of using nano-fertilizers.

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## *In vitro* klijanje i vijabilnost polenovih zrna graška nakon primene organskih nanođubriva

### REZIME

Cilj istraživanja bio je da se proceni uticaj dva organska nanođubriva, Lithovit i Nagro, na *in vitro* klijanje, dužinu polenove cevi i vijabilnost polena *Pisum sativum* L cv. Pleven 4. Uticaj njihove primene bio je visok, odnosno viši od vrednosti u kontroli (44.2 % za klijanje polena i 47.23 % za izduživanje polenovih cevi), kao i od vrednosti za kontrolno organsko đubrivo Biofa na bazi algi (17.5 i 27.9 %, respektivno). Polenova zrna su inokulisana u četiri medijuma. Medijum sa 15% saharoze i 1% agara pokazao je najveći stimulatívni uticaj na polenova zrna graška. Vijabilnost polena, procenjena bojenjem sa 1% karmin crvenom, bila je u granicama 74.72-87.97%. Najviša vijabilnost polenovih zrna zabeležena je nakon primene nanođubriva Nagro.

**Cljučne reči:** Organska đubriva; Polen; Klijanje; Grašak