Short Communication

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HOW ENSURE *IN VITRO* POLLEN GERMINATION OF CULTIVATED FLAX AND ITS WILD RELATIVES

SUMMARY

Until recently, flax was considered a crop whose pollen does not germinate well under artificial conditions. At the same time, rapid acquisition of information on pollen quality is important in many studies. Two linseed varieties and its three wild relatives with n = 15 were used as pollen sources. The basic artificial nutrient medium for pollen germination consisted of 200 mg/L boric acid and 200 mg/L calcium chloride. Experimental treatments additionally included polyethylene glycol of various molecular weights and sucrose. The media with polyethylene glycol 2000 in concentrations of 20-30 % ensured good pollen germination of cultivated flax, showing about 40% of pollen grains with normal pollen tubes. For wild species pollen, 20 % osmotic concentration was more preferable. The addition of sucrose to a medium with polyethylene glycol 2000 disadvantageously influenced pollen germination of cultivated flax and did not affect this indicator for wild species. When polyethylene glycol of higher molecular weight was used in a nutrient medium as an osmotic, the germinated pollen grains usually had burst pollen tubes or did not germinate at all.

Keywords: cultivated flax, wild species, pollen, *in vitro* germination, osmotic agent

INTRODUCTION

Flax (*Linum usitatissimum* L.) is one of the oldest cultivated plants, whose products have long been used for a variety of human needs. Two long-standing main directions of using the products of this plant have formed two different industrial crops – fiber flax and oil flax (linseed). Now each of these crops is grown on millions of hectares and a separate breeding is carried out for each of them. Some wild annual species with n = 15 of the *Linum* genus are actively involved in the breeding process when creating new varieties as the donors of

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many morphological, physiological and biochemical traits for cultivated flax (Jhala *et al.,* 2008; Lyakh and Soroka, 2008).

A very broad use of cultivated flax and its wild relatives provides for knowledge of the quality of pollen, produced by the plant. Pollen quality is important in many aspects. It influences seed set and ensures high seed yield. Again, when breeding programs for inter- and intra-species hybridization are laid out, it is necessary to evaluate pollen quality. Pollen could be also used to estimate some characteristic responses of mature plants to biotic and abiotic stresses at early stages of development and at the same time pollen germination could serve as a sensitive and simple bioassay for environmental quality. Thus, *in vitro* pollen germination technique was used by Hebbar *et al.* (2018), Karim Sorkheh *et al.* (2018) to screen genotypes tolerant to high temperature or to elucidate the genotype response at various temperature levels. In the studies of Lyakh *et al.* (1998), Patil *et al.* (2006), Lyakh and Totsky (2014) pollen was used to select valuable genotypes at the level of male gametophyte as well.

The ability of pollen to grow and germinate on an artificial medium allows estimating its quality fast and effectively. According to Golubinsky (1974) pollen of some plants needs only water to germinate. Pollen of most plant species, however, requires for its germination the presence in the nutrient medium of an osmotic agent, represented most often by sucrose (Baloch *et al.,* 2001; Liu *et al.,* 2013). Analyzing the composition of nutrient media for *in vitro* pollen germination in more than 800 plant species, D. Tushabe and S. Rosbakh (2021) note that sucrose is a component of such media for 89 % of species belonging to various genera and families. According to these and other authors, the other two most commonly encountered components of culture media are boric acid and calcium (Wang *et al.* 2003; Ahmad *et al.* 2012; Wani *et al.* 2020). In some plants, success in germinating pollen *in vitro* is ensured by changing the pH-value (Zaman, 2009; Acar *et al.* 2010). At the same time, it is known that not only sucrose can act as an osmotic agent. Instead of sucrose polyethylene glycol of the certain molecular weight can be added to a germination medium. Sometimes artificial nutrient media for pollen germination can include sucrose in addition to polyethylene glycol (Jayaprakash, 2018).

In *Linum usitatissimum*, separate attempts were undertaken to germinate pollen *in vitro* (Pandey and Kumar, 2013). However, the nutrient medium they used, in which sucrose acted as an osmotic, did not favor normal pollen germination. To date, for the pollen of both cultivated flax and its wild relatives, the nutrient medium, which ensures the emergence of properly-shaped pollen tubes during pollen germination, has not yet been developed. In this respect we supposed that polyethylene glycol could be used as an osmotic instead of sucrose taking into account that it promotes pollen germination in some species. The purpose of this study was to select for the basic nutrient medium, containing boric acid and calcium chloride, an osmotic agent that provides successful germination of pollen in *Linum usitatissimum* L. and some wild species which easily cross with the cultivated flax.

MATERIAL AND METHODS

The research were conducted at the Institute of Oilseed Crops of the National Academy of Agrarian Sciences of the Ukraine (IOC NAAS) during 2020 year. Two linseed varieties and three wild species of the *Linum* genus with *n* = 15 (*L. angustifolium* Huds., *L.bienne* Mill., and *L. hispanicum* Mill.) from the collection of IOC NAAS were used in the studies as pollen sources.

Boric acid and calcium chloride at the concentration of 200 mg/L, as the most frequently included chemicals for *in vitro* pollen germination, were mandatory components of all tested media (Jayaprakash, 2018). As an osmotic, polyethylene glycol (PEG) of various molecular weights or PEG in combination with sucrose were added to the basic medium containing boric acid and calcium chloride. The following nutrient media, differing in osmotic content, have been tested: PEG 2000, 20 % (a), PEG 2000, 30 % (b), PEG 2000, 30 % + sucrose, 5 % (c), PEG 2000, 30 % + sucrose 15 % (d), PEG 20000, 30 % (e), PEG 20000, 5 % (f).

Pollen was collected at early hours from 7 to 9 a.m., out of 20-40 flowers. Freshly collected pollen was germinated for 3-4 hours in a drop of an artificial medium placed on a slide at the temperature of 25±1°C in the dark. After that, the pollen was viewed under a light Leica microscope (Germany) with a 20^X objective. Pollen grains were counted as germinated if the pollen tube length was more than a pollen grain diameter. Pollen grains with not-burst (normal) and burst tubes were recorded separately. In each 5 replications of each treatment from 300 to 400 pollen grains were analyzed (Lyakh and Soroka, 2008).

The results of the experiments were analyzed statistically at the IOC NAAS and significance level was calculated applying a *t*-test, according to Wasserman (2005).

RESULTS AND DISCUSSION

A starting medium consisted only of boric acid and calcium chloride dissolved in distillate water. Since flax pollen did not germinate on such a medium, it was decided to supply it with an osmotic agent. In the preliminary experiments the liquid medium where only sucrose was used as an osmotic agent showed to be ineffective for pollen germination of the *Linum* species under study. The same adverse effect was observed when an agar-agar was added to the medium to solidify it. If the liquid medium contained PEG instead of sucrose in the concentration of 10 %, single pollen grains started to germinate.

On all the nutrient media tested in the experiment, pollen grains with both normal and burst pollen tubes were detectable among the germinated pollen. Although number of normal tubes in most cases considerably exceeded number of burst tubes, the ratio of both was significantly influenced by the composition of the nutrient medium.

Table 1 shows that a medium containing polyethylene glycol-2000 as an osmotic in the concentration of 20 % ensured a sufficiently good germination of the pollen for the species under study. The percentage of pollen grains with

normal (not burst) pollen tubes ranged from 39.1 % in *L. usitatissimum* to 22.7 % in *L. bienne*. The elevation in concentration of PEG 2000 from 20 % to 30 % reduced this indicator for wild species and did not affect pollen germination in cultivated species.

Table 1. Influence of polyethylene glycol 2000 concentration on pollen germination in *Linum usitatissimum* and its close wild species

Species	Polyethylene glycol 20 %		Polyethylene glycol 30 %	
	Normal pollen tubes, %	Burst pollen tubes, %	Normal pollen tubes, %	Burst pollen tubes, %
L.usitatissimum	39.1±2.77	25.1±2.47	38.5±2.74	21.0±2.29
L.angustifolium	44.8±2.80 [a]	7.6±1.49	8.3±1.59	2.3±0.87
L.bienne	22.7±2.43 [a]	5.5±1.33	9.9±1.82	2.2±0.88
L.hispanicum	24.3±2.66 [a]	6.9±1.57	12.1±1.95	3.8±1.14

[a] – differences between treatments are significant at the 0.1 % level of significance

It should be also noted that on both mentioned media the number of grains with burst pollen tubes was significantly smaller than the amount of normally germinated pollen grains in all species. However, if in wild species the proportion of pollen grains with burst pollen tubes was relatively small (15-20 % of all germinated grains), in cultivated flax *L. usitatissimum* it accounted for about 40 % of all pollen capable of germination.

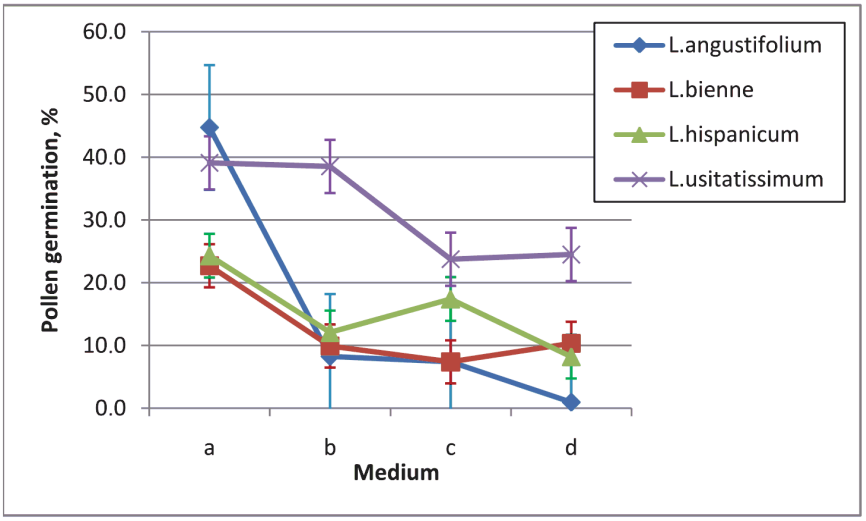

Fig. 1. Influence of PEG 2000 concentration and sucrose addition on the pollen germination of *L. usitatissimum* and its wild relatives with n = 15

Addition to the nutrient medium with PEG 2000 of such an osmotic agent as sucrose adversely affected pollen germination in *L. usitatissimum* (Fig. 1). The inclusion of sucrose at the concentration of 5 % and 15 % to a medium with PEG

30 % reduced the number of pollen grains with normal pollen tubes in this species by almost half. At the same time, in the closest wild relatives, the addition of sucrose to the medium with PEG 2000 did not adversely affect pollen germination.

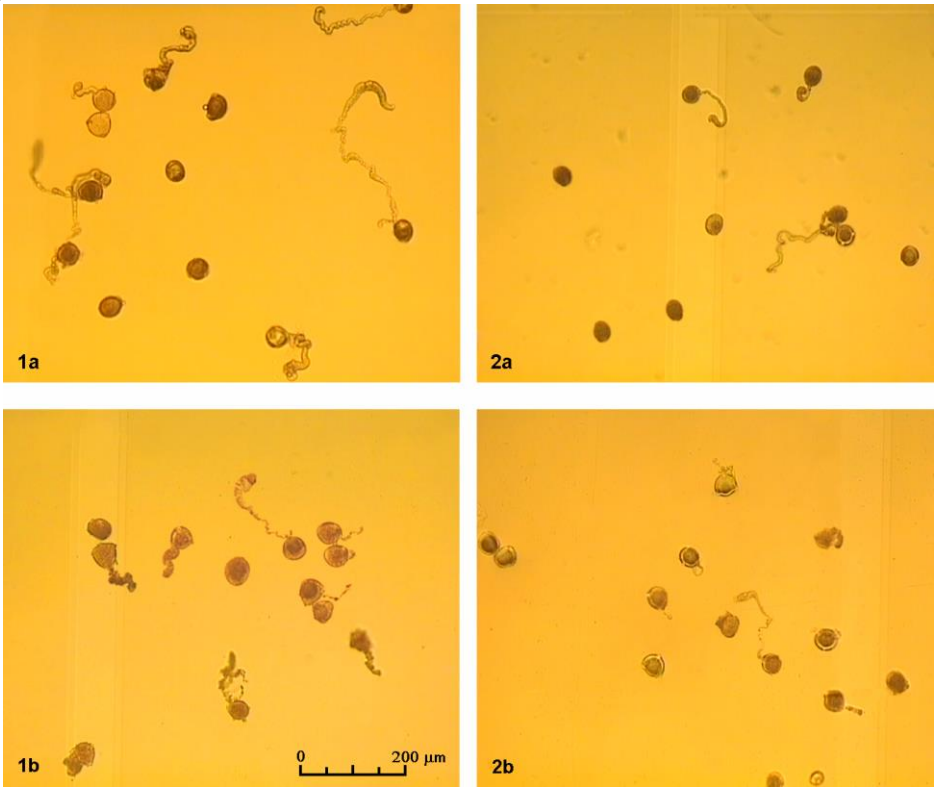

Fig. 2. Pollen germination of *L. usitatissimum* (1) and *L. hispanicum* (2) on the media with PEG of different molecular weight: (a) PEG 2000, 30 % (b) PEG 20000, 5 %

In addition to PEG 2000, PEG of higher molecular weight was tested as an osmotic in two concentrations – 30 % and 5 %. When the medium included PEG 20000 at the concentration of 30 %, pollen of all the studied species failed to germinate. Fig. 2 demonstrates the effect of PEG 20000 at the concentration of 5 % on pollen germination in *L. usitatissimum*, showing the large proportion of pollen grains with burst pollen tubes. When pollen was cultivated on the media, containing PEG 2000 at the concentration of 30 %, pollen grains formed predominantly normal pollen tubes.

Thus, a nutrient medium containing, in addition to boric acid and calcium chloride, an osmotic agent in the form of polyethylene glycol 2000 at the concentration of 20-30 %, can be proposed for evaluating the viability of pollen in cultivated flax and its closest wild relatives. Although in such a medium some of the germinated pollen has burst pollen tubes, the proportion of pollen grains

with normal pollen tubes is high enough to quickly and efficiently assess the quality of the male gametophyte or conduct some sort of selection at this level.

It is known that nutrient media in which sucrose is used as an osmotic agent are quite suitable for the germination of pollen from many plant species, including such well-known ones as, for example, tomato or maize (Jayaprakash, 2018). A similar medium involving only sucrose as an osmotic agent was also tested on flax (*Linum usitatissimum*). However, the abundance of burst and severely deformed pollen tubes developed under such conditions does not allow to assess the viability of pollen of this species in a proper way (Pandey and Kumar, 2013). Some experiments are known where sucrose was successfully combined with another osmotic agent (Jayaprakash, 2018). Thus, for the germination of sunflower pollen a nutrient medium was developed which included 15 % sucrose and 30 % polyethylene glycol 6000 (Keshava Murthy *et al.*, 1994). At the same time, our studies on flax demonstrated that addition of sucrose together with polyethylene glycol either significantly reduced pollen germination, as in cultivated flax, or did not positively affect the ability of pollen to germinate *in vitro*, as in the three wild flax homostyle species. In any of those cases, the addition of sucrose to the nutrient medium simultaneously with polyethylene glycol did not stimulate pollen germination and indicates the inefficiency of this osmotic agent for germination of pollen of any flax species.

It should be also noted that in our research polyethylene glycol at the concentration of 20 % and 30 % was equally effective for the germination of cultivated flax pollen, while the pollen of all three wild flax species reacted negatively to the presence of this osmotic agent in the nutrient medium at the concentration of 30 %, compared with 20 %. It can be assumed that those differences in the reaction of pollen are due to the different osmotic potential of pollen grains of wild species and cultivated flax, which is a consequence of their relatively asymmetrical morphological and physiological features (Jhala *et al.*, 2008).

CONCLUSION

Despite the fact that there are different methods for assessing the quality of pollen - from its germination *in vivo* on the stigmas of pistils to staining with various dyes – *in vitro* pollen germination on a nutrient medium is considered the fastest and most reliable. The revealed ability of pollen of cultivated flax and its closest wild relatives to germinate under artificial conditions makes it possible to control more effectively the success of crosses and determine pollen productivity, to develop methods for evaluating and selecting valuable genotypes at the microgametophytic level, as well as to carry out a number of other manipulations associated with the reproductive system of this important agricultural culture.

As follows from the results of the presented studies, the medium for *in vitro* germination of flax pollen, in addition to the standard boric acid and calcium chloride, commonly used in work with other plant species, requires the presence of an osmotic agent. Polyethylene glycol with a molecular weight of

2000 can act as such an osmotic agent. At the same time, while both concentrations of this osmotic (20 % and 30 %) are quite acceptable for cultivated flax, only 20 % concentration of polyethylene glycol is suitable for annual wild flax species. The use of sucrose together with polyethylene glycol not only does not improve the germination of flax pollen grains, but often inhibits this process.

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