Cleared-ovule method used for early embryo development in flax observation (*Linum usitatissimum* L.)

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Abstract
Present study aimed to determine the possibility of cleared-ovule technique adaptation for early embryo development in Linum (Linum usitatissimum L.) observation. This method was primarily introduced in cereals by Ponitka and Ślusarkiewicz-Jarzina [1]. Flax and linseed seed capsules were sampled after 10 and 15 days since the beginning of flowering. In the ovules isolated, different development stages of embryos were observed. The results obtained suggest, that cleared-ovule technique may be easily applied, not only for Linum embryo development observation, but also as an alternative method for time-consuming and expensive paraffin-embedded technique.

1. Introduction

Linum (*Linum usitatissimum* L.) is a valuable material for economy and human needs. Being the only source of natural cellulose fibres, Linum is significant industrial plant of moderate climate countries. It has its application not only in textile and building industry, but also in feed components production. On the other hand, linseed is used in oil, paint, pharmaceutical, cosmetic and food industry [2]. Recently, this species grew to become the main focus in biotechnological research, mainly because of the new possibilities of flax fiber application for functional nano- and micro-materials production. Since these fibers, apart from cellulose, contain also antioxidant compounds, they can be easily applied for medical dressings and chirurgical threads production [3, 4]. Known for years, cleared-ovule technique,
is commonly used in embryology of different species [5]. In 1979, Young et al. introduced a cleared-ovule technique,

involving methyl salicylate [6], which enables the embryo observation without disturbing the ovule structure. This method is less time- and work consuming than commonly used paraffin-embedded technique. The present study aimed to adapt the cleared-ovule method, according to Ponitka and Ślusarkiewicz-Jarzina [1] research conducted in 2004, which was easily introduced in cereals. Adjusting this method for Linum may cause the stadium of embryo development estimation easier and more effective. Moreover, it can be very useful i.a. for pollination effectiveness assessment by estimating the number of ovules with embryos in seed capsule in *in vitro* cultures. Being used in practice for isolated embryo cultures, this method can be directly applied for breeding practice and cause the new, economically valuable cultivars obtaining.

2.Material and methods

The plant material used for present study was linseed and flax cultivars (*Linum usitatissimum* L.). The seeds of cultivars

analyzed derived from Centre for Genetic Resources the Netherlands, Wageningen, Holland and from the Institute of Natural Fibres and Medicinal Plants in Poznan (INF&MP) (Tab. 1). In order to observe the embryos of five linseed and flax cultivars, after 10- and 15 days since the beginning of flowering, the seed capsules from the whole plant were sampled and fixed, what enabled obtaining the plant material of different levels of embryo development (fixing agent – mixture of 40% formalin, chloroform, 70% ethyl alcohol, 5:5:90 respectively). The fixed material stored in 70% ethylene alcohol. The ovules development and presence of embryos were analyzed, using cleared-ovule method [1] with some alterations. Hence, from the fixed seed capsules, some embryos were isolated, which were afterwards dehydrated by ascending order of 70%, 80%, 90% and 100% ethylene alcohol. The ovules were placed in 70% ethanol for a half of an hour, than in 80 and 90% ethanol for two hours. Plant material placed in 100% ethanol was left till next day. The following day, the ovules were placed in fresh 100% ethanol for one hour. Dehydrated ovules, were placed in methyl salicylate and ethylene alcohol mixture within two hours in solutions 1:1, 3:1 and eventually put into pure salicylate twice after an hour. From the plant material prepared as described above, preparations were made, and the observations of ovules and embryos were carried out, using the Nikon Phase Contrast Microscope.

Table 1

The comparison of Linum cultivars (*Linum usitatissimum* L.), used in the study, including the utility type and the country of origin.

Genotype No. According to Gene Banks and INF&MP	**Cultivar**	**Country of Origin**
Linseed		
21202	Arrow	USA
21203	Baladi	Egypt
21248	Cypern	Cyprus
21291	Victory	USA
Flax		
19341	Ballinacura	Ireland
19345	Caxias	Brazil
INF00778	Artemida	Poland
INF00811	Atena	Poland
INF00779	Modran	Poland
INF00780	Selena	Poland

2. Results

The cleared-ovule method enabled the expose of embryos, even in early, single cell stages. Every fixed seed capsule ovule from each plant was analyzed, to compare most precisely development stages of every embryo. As far as every cultivar is concerned, 40-60 ovules deriving from one plant were examined. Between the cultivars and the utility types examined, some dissimilarities concerning flowering time were observed (Tab. 2). The flowering of the Arrow cultivar was the earliest, and of the cultivar Selena was the latest. Despite the discrepancies concerning the flowering time between the cultivars and utility types examined, in most cases the embryos observed were at the same stage of development after 10 and 15 days since the beginning of flowering. Embryos of most of the cultivars, fixed after 10 days since the beginning of the flowering, were in globule stadium (Figs. 1-4), except the Victory
(Fig. 5) and Artemida cultivar, of which the embryos fixed after this time, were in early torpedo stadium. According to all Linum cultivars analyzed, using the preparations made from plant material fixed after 15 days since the beginning of the flowering, embryos with apparent radicles were noticed (almost mature embryos) (Fig. 6). One seed capsule included ovules with embryos at the same development stage. Within the whole plant, the seed capsules differed from each other over size. However, as far as the isolated embryos of the smallest seed capsules are concerned, no embryos were observed. In turn, embryos at the

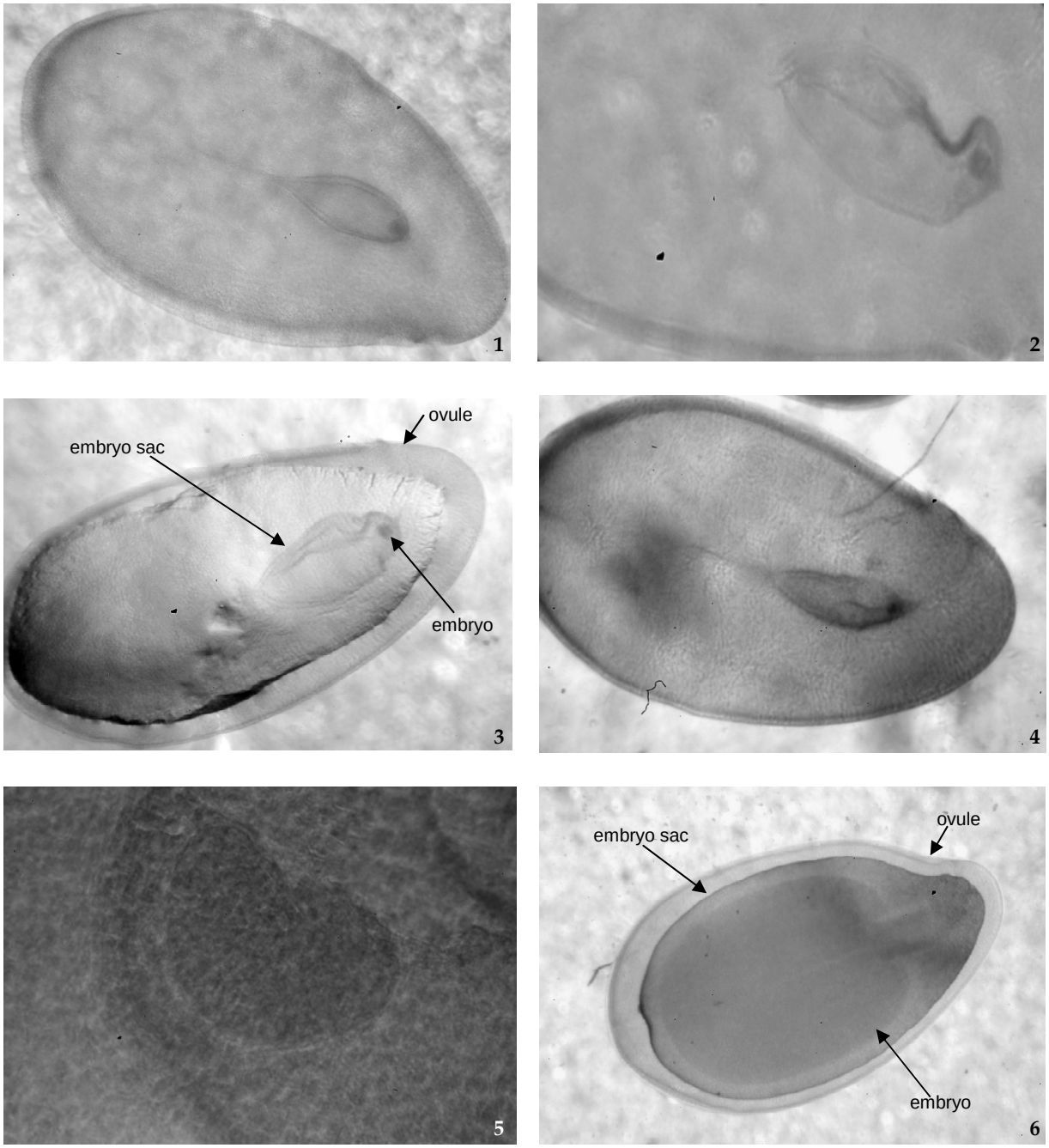
same development stage - globule stage, early torpedo or with apparent radicle, were reported for bigger seed capsule ovules.

Table 2
Development stages of Linum (*Linum usitatissimum* L.) cultivars embryos, after 10 and 15 days since the beginning of flowering, including the time of flowering.

Cultivar	Earliness of flowering	Embryo development stadium	
		After 10 days since flowering	After 15 days since flowering
Linseed			
Arrow	10.06.2009	globular stadium	almost mature
Baladi	14.06.2009		
Cypern	15.06.2009		
Victory	12.06.2009	early torpedo	
Flax			
Ballinacura	17.06.2009	globular stadium	almost mature
Caxias	17.06.2009		
Artemida	22.06.2009	early torpedo	
Atena	20.06.2009	globular stadium	
Modran	20.06.2009		
Selena	23.06.2009		

3. Discussion

The cleared-ovule technique enables the embryos observation, even at their very early stages of development. Adjusting such method became an alternative for time- and work consuming paraffin-embedded method without the necessity of ovule cross section. The results obtained, indicate that after some alterations, the Ponitka and Ślusarkiewicz-Jarzina [1] technique for cereal, can be easily applied for Linum (*Linum usitatissimum* L.) embryo development observation. However, this method is still in need for further examination. In the present study, methyl salicylate properties were used, although, according to literature, in cleared-ovule technique, other reagents can also be used [7]. These methods are commonly used, not only for basic research, characterizing embryology of certain species [8], but also for degree of pollination in crossbreeding estimation [1], defining the grasses apomixes [6] and in research on such species as *Medicago*, *Solanum*, *Ranununculus* and *Lilum* [9]. Furthermore, it was possible to adjust this method for *Brassica* pollination effectiveness in crossbreeding estimation [10], which makes it very useful from breeding point of view. Similar application can be made in Linum. Therefore, it is necessary to conduct controlled crossbreeding, in order to determine the exact moment of pollination, which however, was not the issue in this study. Only then, the development stages of ovule embryos could be fully described, with simultaneously indicating if the development rate is actually the same between the plants examined. Within the cultivars, only the three embryo development stages were observed, hence more ovules differing in size need to be examined for obtaining the full piece of data for this species. In addition, determining the exact embryo development stage and connecting it with the size of the seed capsule, could introduce the method to isolated embryos culture conducting in *in vitro* conditions.



Figs. 1-5. The ovules of Linum cultivars (*Linum usitatissimum* L.) after the application the cleared-ovule metod with methyl salicylate, after 10 days since flowering, Fig. 1 – Atena cultivar– globule stage embryo Fig. 2– Artemida cultivar - globule stage embryo, Fig. 3– Arrow cultivar - globule stage embryo , Fig. 4– Baladi cultivar - globule stage embryo Fig. 5– Victory cultivar– the early torpedo stage embryo, Fig. 6– Atena cultivar– embryo with radicle and one cotyledon, after 15 days since flowering.

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