Morphophysiology and Cryopreservation of Seeds of Dendrobium Nobile Lindl. (Orchidaceae) at Different Stages of Development

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Research Article

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Abstract

We studied the seed maturation of *Dendrobium nobile* Lindl to establish the optimal stage for green pod culture and long-term storage in liquid nitrogen. It was found that the immature seeds can germinate *in vitro* starting from 3 months after pollination (MAP) but only develop up to the stage of embryo swelling or protocorm without rhizoids. The maximum staining of embryos with vital dyes FDA and TTC occurs at 4 and 5 MAP that corresponds to the release of suspensor beyond the embryo sac. Embryo staining does not correlate with germination capacity and seed viability in cryogenic storage. The immature seeds acquire resistance to freezing and drying in airflow at the age of 6 MAP. The time of technical maturity for green pod culture and cryopreservation may be reduced from 12 MAP to 6–7 MAP.

Key Message

The immature seeds of Dendrobium nobile acquire resistance to freezing and long-term storage in a cryobank at the age of 6 MAP.

Main Text

The ongoing interest in orchids cultivation is explained not only by the ornamental features of the flowers but also by diverse biologically active compounds, produced in their stems and leaves. *Dendrobium nobile* Lindl. are widely used in traditional eastern and modern western medicine.

One of the ways to optimize the *in vitro* orchid cultivation may be a green pod culture in which immature seeds are germinated (Vasudevan and van Staden 2010; Udomdee et al. 2014). Collection orchids cultivated in the greenhouse are severely depleted during fruiting, therefore, it is desirable to reduce the maturation period.

However, the requirements for seed germination *in vitro* and cryogenic storage are different. Immature seeds that contain a lot of water in cells are better for *in vitro* germination, and mature dried seeds are better for cryogenic storage.

The work aims to determine the time of morphophysiological maturity of *Dendrobium nobile* seeds for the green pod culture and long-term storage in liquid nitrogen.

Plants of *Dendrobium nobile* Lindl. (Orchidaceae Juss.) (Fig. 1A) were cultivated in the greenhouse with a moderately warm temperature regime (day 18–26 ^{o}C, night 14–18 ^{o}C) at a relative humidity of 60–75% and natural light. The flowering of different clones continued from November to March. The fruits were obtained by artificial allogamous pollination. The age of the seeds and ovules was calculated in months after pollination (MAP).

Germination ability was evaluated before and after cryogenic storage of seeds. The fruits were surface sterilized with 70% ethanol. The seeds were extracted with a sterile tool. More mature seeds (7–12 MAP)

were sterilized in 5% calcium hypochlorite for 15 min and washed with sterilized distilled water. Samples were germinated for 2 months in Petri dishes on a hormone-free 1/2 MS basic medium in the dark at 25 °C. The percentage of protocorms was calculated in 3 repetitions of 100–200 seeds in each. The stages of development of protocorms were determined by the Vasudevan and van Staden (2010) method: 1. imbibed seeds, swollen, still covered by testa (= viable seeds) (Fig. 1D, E); 2. enlarged seeds without testa (=germination) (Fig. 1C); 3. protocorms with rhizoids (Fig. 1D); 4. protocorms with pointed shoot apex and rhizoids (appearance of shoot apex) (Fig. 1E). The stages of seed germination were recorded using a Keyence VHX-1000E light microscope.

The water content was determined by the weight loss of seeds (50 mg in 2 replicates) after drying to constant weight at 95 °C.

For cryogenic storage, the samples were partially dried in a sterile airflow at room temperature and 40-60% relative humidity for 2–6 h in the laminar box. The drying stopped when the sample weight was reduced by 60–70%. The dehydrated seeds in sterile cryo-vials (Nunc, USA) were immersed in liquid nitrogen for 1 month.

The viability of seeds was also determined by vital staining with 0.005% fluorescein diacetate (FDA, Sigma-Aldrich, USA) or 1% 2,3,5-triphenyltetrazolium chloride (TTC). The samples were stained on a glass slide in the dark wet chamber for 2 hours, washed several times with distilled water and mounted in 50% glycerol. Samples were investigated with Olympus FV1000D confocal microscope and Axioplan 2 light microscope. The fluorescence was excited with violet (405 nm) and blue (473 nm) lasers at 50% power. The signal was recorded in blue (425–460 nm), green (485–530 nm) and red (560–660 nm) channels.

D. nobile fruit is a dehiscent capsule (Fig. 1B). The time of seed ripening is 12–13 MAP.

The archesporial cell, the inner integument initials and megasporogenesis were noted at the age of 2 MAP. Maturation of the embryo sac and fertilization occurred at 2.5–3 MAP. A few-celled (2–4 cell) embryo formed at 3–3.5 MAP. The suspensor at this stage was still within the embryo sac (Fig. 2A). The developmental stages from zygote to multicellular embryo were found in fruits at 4–6 MAP. We observed the variability due to the uneven development of ovules, as well as the abortive ovules without an embryo (Fig. 2E).

At the fertilization stage (2–2.5 MAP), the ovule had two double integuments surrounding the embryo sac. The micropylar end turned towards the placenta (anatropic ovule). At the early embryogenesis stage (3 MAP), the inner part of the inner integument is covered with a cutinized layer (Fig. 2A). This layer had a reddish-brownish autofluorescence. It was black-brown when stained with FDA (Fig. 2B) and was almost invisible when stained with TTC (Fig. 2E). The cutinized shell was thin at the micropylar end and thicker at the chalazal end (Fig. 2A).

Seeds with suspensor inside and outside the embryo sac were identified on 4 MAPs (Fig. 2B, C). The first category included smaller multicellular embryos with vesicle suspensor. The second category included

larger multicellular embryos with a branched unicellular suspensor. Living cells of the inner lining died at 5 MAP (Fig. 2C). Suspension desorption and the formation of a multicellular autonomous embryo were observed at 6 MAP (Fig. 2D). The protoplasts of the inner layer of the outer integument died at 7 MAP, and the formation of a full-fledged seed coat from the outer layer of the outer integument occurred at 8 MAP.

The table shows the parameters of seeds before immersion in liquid nitrogen and after cryogenic storage. The immature seeds at the initial stages of embryogenesis poorly retained moisture. Large water losses are associated with intensive morphogenetic processes, in particular, the suspensor formation and its exit outside the embryo sac. The immature seeds stabilized the ability to retain moisture after suspensor desorption.

During the germination of immature seeds at the stage of the first embryonic divisions (3–3.5 MAP), the embryos swelled and formed protocorms without rhizoids (Fig. 1C). No further development occurred.

Seeds at 4–5 MAP retained germination after drying (16%) but lost germination after freezing. At 6 MAP, 30% of the seeds germinated *in vitro* after dehydration in airflow and only 10.7% of the seeds germinated after storage in liquid nitrogen. At 7 MAP, 81–94% of seeds acquired cryostability and retained their germination after cryogenic storage. Over 80% of seeds had stable germination both after drying and after cryogenic storage until full ripening and fruit opening at 12 MAP.

The earliest time that immature *D. nobile* seeds can be successfully stored in liquid nitrogen in a viable state and germinated if necessary is 6 MAP. A visual sign of seed maturation is the appearance of a yellow colour. The proportion of protocorm formation and further organogenesis is higher in seeds older than 6 MAP (Fig. 1E, F). However, due to many seeds in the fruit, their relatively low germination after cryogenic storage is not critical.

Fig. 2E, F shows the results of the TTC tests. The proportion of FDA or TTC-positive seeds and the staining dynamics did not correlate with the *in vitro* germination (Fig. 3, Table). The largest proportion of stained embryos was observed at 4–4.5 MAP. At this stage, the suspensor extends beyond the embryo sac. Vital dyes stained both less developed embryos with a rounded suspensor inside the embryo sac and embryos with a large branching suspensor outside it. Cutinization of the inner layer of the inner integument after fertilization probably not only fails to protect the embryo from excessive dehydration but is also permeable to vital dyes.

The proportion of coloured embryos decreased as the seeds matured. Staining decreases after suspensor desorption at 6 MAP. The cells of the inner integument died, their periclinal walls became denser, forming an impermeable membrane around the embryo. Thus, the multicellular embryo acquired autonomy, and the percentage of seed germination and cryostability increased.

The difficulties of seed reproduction of orchids in culture are associated with the morphological features of seeds (Rasmussen 1995).

In *D. nobile* and its hybrids, the ability of seeds of different ages to germinate *in vitro* varies in different authors from 3–4 MAP (Vasudevan and van Staden 2010; Udomdee et al. 2014) to 6 MAP (Poddubnaya-Arnoldi 1964). In our work, immature seeds germinated *in vitro* at the age of 3 MAP. Early stages of development of the ovule were observed during this period, but mainly the formation of the embryo sac and fertilization.

Our study confirmed the ability of *D. nobile* seeds to germinate at 3 and 4 MAP, but the development stopped at the stage of protocorms without rhizoids.

Vital dye staining is often used to assess the viability of orchid seeds. The methods using TTC and FDA were described by Wood and Pritchard (2004) and Sawma and Mohler (2002) respectively. These tests are often used due to a simple assessment by counting coloured embryos. However, the time of seed staining, as well as the pre-soaking in distilled water or other substances (for example, when sterilized with $Ca(OCl)_2$), significantly affect the intensity of staining and, therefore, distort the results of the tests (van Waes and Debergh 1986; Vujanovic et al. 2000). Our study showed that FDA and TTC staining did not correlate with the germination ability of *D. nobile* seeds. The increase in the number of FDA-stained embryos at the stage of active embryogenesis (4 MAP) in our work corresponds to the data of the authors who used TTC (Vasudevan and van Staden 2010).

Compared to most crop seeds, mature seeds of epiphytic orchids are short-lived. Today, the most long-term preservation of orchid seeds is possible in cryobanks at liquid nitrogen (–196°C) (Engelmann 2004).

Optimal seed moisture is crucial for viability. Several techniques are used to prepare immature seeds for freezing (Engelmann 2004; Hirano et al. 2005; Merritt et al. 2014). In our work, the seeds were dried in sterile airflow. In this case, the germination of seeds is influenced not only by freezing but also by dehydration (Makeen et al. 2005).

Thus, the fruits of *D. nobile* fully ripen and open at the age of 11–12 MAP, the seeds acquired resistance to freezing and drying in the airflow at the age of 6 MAP and the optimal period for cryopreservation of immature seeds is 7 MAP, which reduces the time to achieve technical maturity by five months.

Declarations

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Conflicts of interest/Completing The authors declare that they have no conflicts of interest.

Availability of data and material All data transparency

Code availability Not applicable

Author's contributions The authors took an equal part in writing the manuscript.

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Tables

Table. Germination of *D. nobile* seeds of different ages after drying in airflow, cryogenic storage and two months of *in vitro* culture

Age, MAP	Initial humidity, %	Humidity after drying, %	Initial germination*, %	Germination after drying, %	Germination after cryogenic storage, %
2	92,3	75,5	0	0	0
3	89,2	63,2	21,7±6*	0	0
4	85,4	11,7	19,0±1*	16,0±3*	0
5	86,0	13,1	26,3±1**	single protocorms**	single protocorms**
6	80,1	19,0	33,7±4	30,3±1	10,7±2
7	70,9	17,8	82,3±2	82	81,3±1
8	52,3	29,2	98,3±1	90±1	87,7±1
10	28,1	24,5	94,7±4	90±2	93,7±2
12	43,3	21,1	94,3±2	89±2	89,7±2

The most developed protocorms in the sample: * – protocorms without rhizoids; ** – protocorms with rhizoids; without asterisks – protocorms with rhizoids and apical bud

Figures

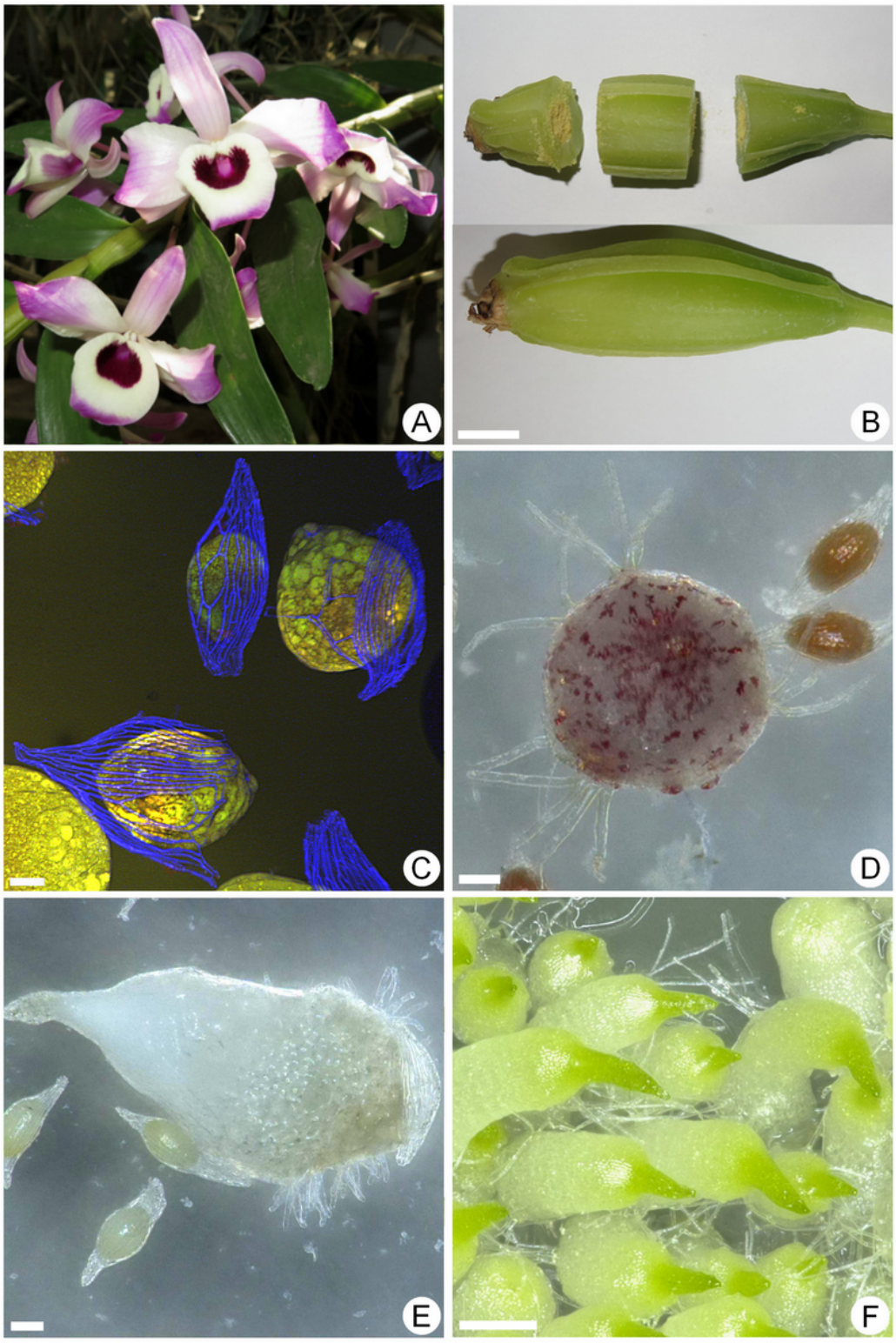

Figure 1

Flowers, fruits and protocorms of D. nobile. A Flowers; B mature fruits at 12 MAP; C enlarged protocorms without rhizoids (FDA, confocal microscopy); D protocorm with rhizoids; E protocorm with pointed shoot apex and rhizoids (cultivation in the dark 4 weeks); F protocorms with pointed shoot apex and rhizoids, germinated 3 weeks in the dark and 1 week in the light. D–F Keyence VHX-1000E light microscopy. Scale bars: B = 1000 µm; C–E = 100 µm; F = 500 µm.

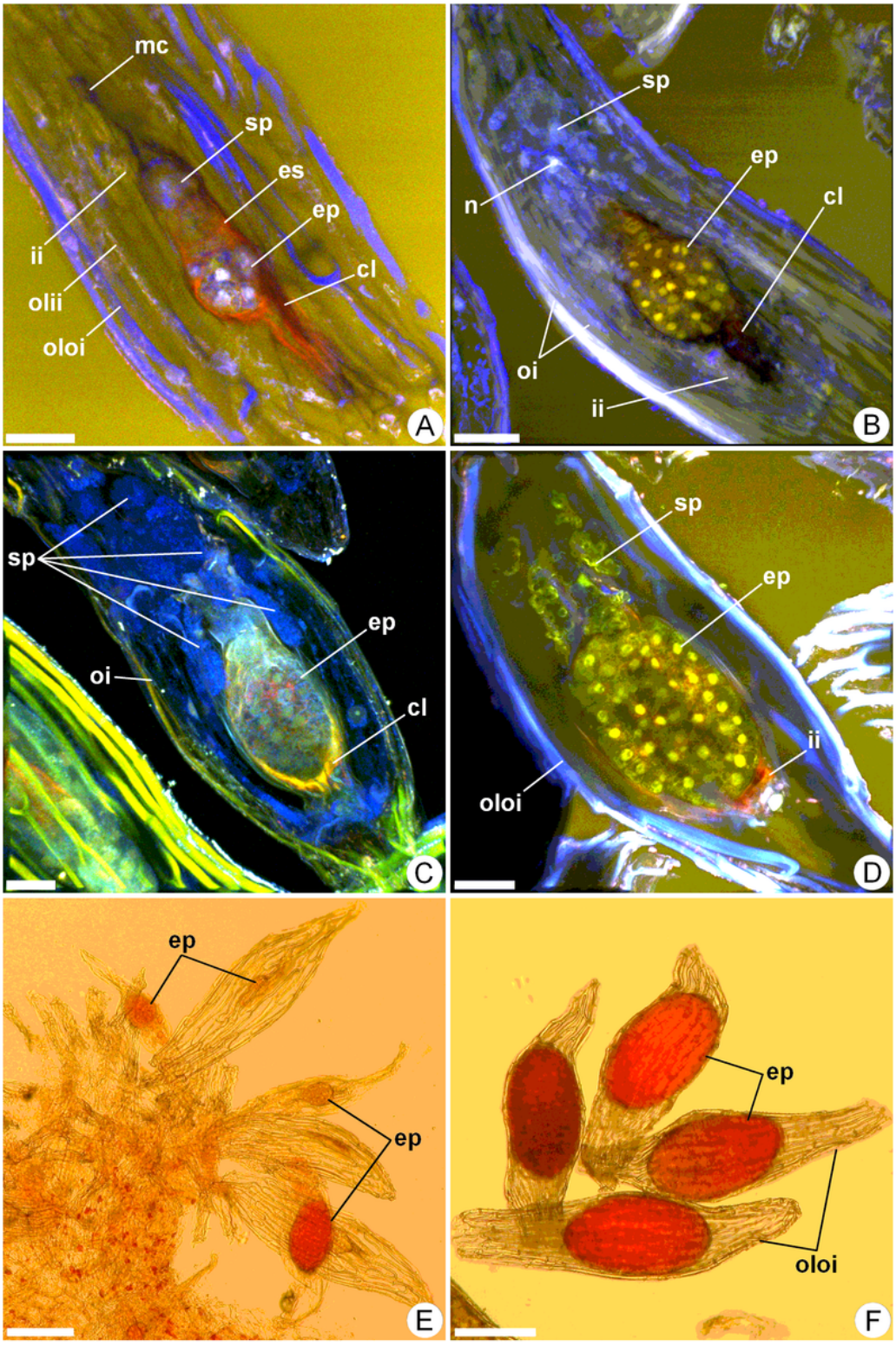

Figure 2

Ovules and seeds of D. nobile. Confocal microscopy: A the suspensor of the few-celled embryo does not extend the embryo sac, the cutinized inner layer of the inner integument fluoresces red-brown (3 MAP) (FDA); B the suspensor extends beyond the embryo sac, the nuclei of the embryo proper fluoresce yellow, the nucleus of the suspensor fluoresces blue, the dark cutinized shell of the inner layer of the inner integument does not fluoresce (4 MAP) (FDA); C the branched suspensor fluoresces blue after elimination

of the inner integument (5 MAP) (Auto); D elimination of the inner layer of the outer integument and the suspensor, nuclei of the embryo proper fluoresce yellow (6 MAP, FDA). Light microscopy (Axioplan 2): E and F the seeds of D. nobile, stained with TTC at 5 MAP (E) and 8 MAP (F). Scale bars: A = 20 µm; B–F = 100 µm; D = 30 µm. cl, cutinized layer; ep, embryo proper; es, embryo sac; ii, inner integument; mc, micropyle; n, nucleus; oi, outer integument; olii, outer layer of inner integument; oloi, outer layer of outer integument; sp, suspensor

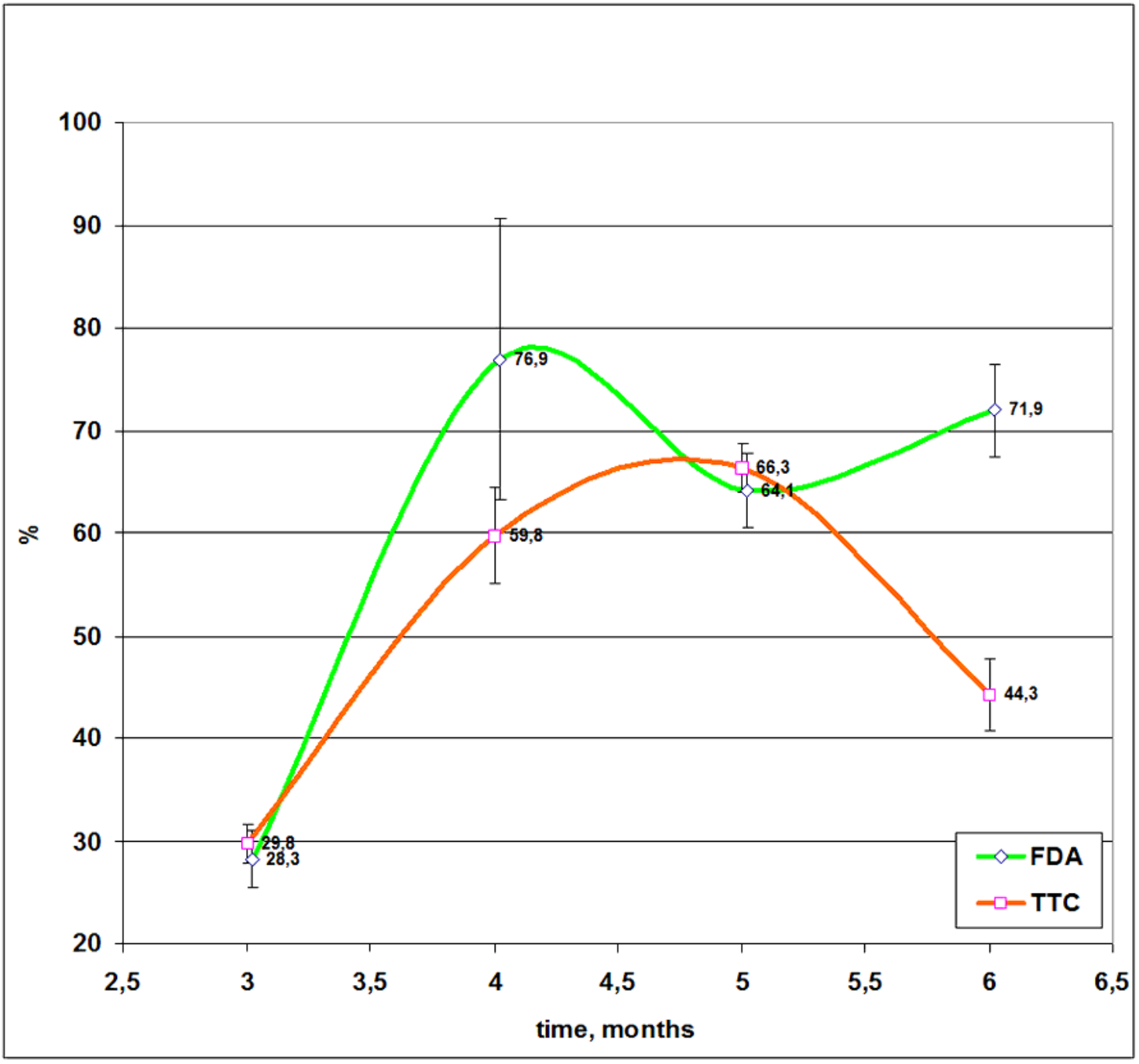

Figure 3

Embryo staining with FDA and TTC vital dyes as a percentage of the number of seeds and ovules with an embryo at the age of 3–6 MAP