Effect of 2,4-D as a Novel Inducer of Embryogenesis in Microspores of *Brassica napus* L.

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Abstract: The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) applied at high concentrations for a short time was investigated as a novel stress for induction of microspore embryogenesis for the first time. *Brassica napus* L. cvs. Topas and Hyola 420 were used as model plants for testing this hypothesis. Microspores were subjected to 2,4-D at 4 concentrations (15, 25, 35 and 45 mg/l) for 15–45 min while the classical heat shock was used as the control treatment. Among 2,4-D treatments in Topas, the highest yield of torpedo-stage embryos was achieved at 15 mg/l 2,4-D for 30 min while more normal plantlets were produced when 2,4-D (25 mg/l for 30 min) was applied to the microspores. In Hyola 420 the results showed a lower number of embryos and normal plantlets at all concentrations of 2,4-D. Although Hyola 420 was almost equally embryogenic as Topas after heat shock treatment, large differences between genotypes (concerning embryogenic response) occurred after 2,4-D treatment. However, the mean number of embryos and regenerants was higher in heat shock as compared to 2,4-D induced stress (one magnitude of order). According to the results obtained, 2,4-D can be introduced as a new stress for induction of embryogenesis in microspores similarly like in zygotic and somatic cells. This novel stress is very important for plant species whose microspores are extremely sensitive to classical stresses.

Keywords: 2,4-D; *Brassica napus*; doubled haploids; microspore embryogenesis; rapeseed

The production of haploid plants from isolated microspores of rapeseed (*Brassica napus*) was reported first by Lichter (1982). *B. napus* L. is frequently used as a model plant for microspore embryogenesis in order to investigate new ideas. In addition, spring rape cultivars (e.g. Topas) are the most responsive rape cultivars to improve and optimize protocols of microspore embryogenesis (Coventry *et al.* 1998).

Stress as a triggering factor is necessary for induction of microspore embryogenesis (reviewed by Shariatpanahi *et al.* 2006a). Stresses widely used for induction of microspore embryogenesis are cold, heat, carbon starvation and colchicine. Heat shock has been used as a trigger to induce embryogenesis in isolated microspores of several species, i.e. rapeseed, wheat, tobacco, eggplant, timothy (Shariatpanahi *et al.* 2006a).

Among different auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) has been applied for induction of somatic embryogenesis the most commonly (Gaj 2004). This

synthetic growth regulator and auxinic herbicide appears to act not only as an exogenous auxin analogue but also as an effective stressor (Gaj 2004). It has been hypothesized that 2,4-D concentration (around 10μM) acts at the same time as an auxin (either itself and/or through endogenous indole acetic acid (IAA) levels) and as a stressor (Feher *et al.* 2002). Auxinic herbicides have been shown to interact with ethylene and abscisic acid (ABA) synthesis, increasing the cellular levels of these hormones (Grossmann 2000; Wei *et al.* 2000). Rodrigues *et al.* (2004) studied the effect of light and 2,4-D (2 and 10 mg/l) on the efficiency of microspore embryogenesis in soybean and found that 2,4-D at a higher concentration could not improve androgenesis. However, a high concentration (40 mg/l) of 2,4-D was employed to induce somatic embryogenesis from immature soybean cotyledons (Bailey *et al.* 1993). Also, Kim *et al.* (2004) reported the induction of embryogenic calli (at the frequency of 53%) in petiole explants of *Hylomecon vernalis* when the explants were cultured on B5 medium supplemented with 13.6μM 2,4-D alone. Leaf protoplast-derived cells treated with 2,4-D could develop into pro-embryogenic cell clusters (Pasternak *et al.* 2002). Sharma *et al.* (2007) found out that the somatic embryogenesis in potato internodal segments could be evoked by 2,4-D-pulse treatment over a wide concentration and duration range. 2,4-D also affects the activity/function of different genes responsible for biosynthesis pathways such as production of auxin, ABA and ethylene (Michaczuk *et al.* 1992; Grossmann 2000; Wei *et al.* 2000; Feher *et al.* 2003; Raghavan *et al.* 2006).

Perera *et al.* (2009) investigated the effect of growth regulators on the efficiency of androgenesis in coconut. It was reported that 2,4-D (100μM) in combination with 1-naphthaleneacetic acid (NAA) (100μM) enhanced the production of calli/embryos. A high 2,4-D concentration (5–6 mg/l) increased embryo production in anther cultures of *Avena sterilis* and *A. sativa* × *A. sterilis* progeny and promoted plant regeneration in genotypes of both species (Kiviharju & Tauriainen 1999).

2,4-D has been widely used for induction of embryogenesis in zygotic cells of *Arabidopsis thaliana* (Raghavan 2005) and somatic cells in more species, e.g. *Hylomecon vernalis* (Kim *et al.* 2004), *Medicago sativa* (Pasternak *et al.* 2002), *Solanum tuberosum* (Sharma *et al.* 2007).

2,4-D has been employed in most anther/microspore culture systems either as an auxin (to elicit rapid cell proliferation and callus formation) or as a co-trigger for microspore differentiation in combination with other pre-treatments (Xie *et al.* 1995; Hoekstra *et al.* 1996; Zheng & Konzak 1999; Rodrigues *et al.* 2004; Shariatpanahi *et al.* 2006b).

The incubation of wheat anthers with 2.0 or 4.0 mg/l 2,4-D in an induction medium for 10 or 15 days was reported to be sufficient for the initiation of callus development (Zheng & Konzak 1999). However, the reduction or removal of 2,4-D in the medium beyond the initiation phase was essential for plant regeneration from calli. It was also reported that the quality of auxin-free derived calli was poor. It is known that the first cell division during callus induction from anthers occurs within 2 days after inoculation for which 2,4-D plays a key role (Reynolds & Kitto 1992). However, for the transition from callus and/or embryo development to plant regeneration, the reduction or removal of 2,4-D is needed since new gene products are required (Zheng & Konzak 1999). The same effect of 2,4-D as an auxin was reported in rice isolated microspore culture (Xie *et al.* 1995) in which 2,4-D was needed for induction of microspore-derived calli. Shariatpanahi *et al.* (2006b) also reported the positive influence of 2,4-D (2 mg/l in induction medium) on the quality of microspore-derived embryos in a wheat isolated microspore-culture system.

The influence of 2,4-D (10 mol/l continuously or for 1–7 days) in combination with mannitol pretreatment of barley anthers/isolated microspores was reported for induction of microspore differentiation (Hoekstra *et al.* 1996). They observed that without mannitol pretreatment no embryogenic type of microspores could be recognized at the moment of microspore isolation, and plating efficiency never reached 1%.

In another report, the combination of 2,4-D (10 mg/l) and light conditions (16h photoperiod at ±2000 lux) was shown to be an inducer of the callogenic response of anther walls and connective tissue in soybean anther culture, however the 2,4-D concentration had no effect on embryogenesis (Rodrigues *et al.* 2004). They reported plasmolysis of microspores in 10 mg/l 2,4-D both in the presence or absence of light, indicating a degradative effect of this auxin at this concentration.

As explained above, there has been yet no report on the induction of microspore embryogenesis by 2,4-D alone. In this paper the use of 2,4-D alone as a trigger to induce microspore embryogenesis was investigated for the first time.

MATERIAL AND METHODS

The spring rapeseed cultivars Topas and Hyola 420 were used in this study. Seeds were germinated on soil in pots in a growth chamber with a temperature of 18–20°C and day length of 16 h. After 2 months, young flower buds with microspores at the late unicellular developmental stage were collected from main and lateral branches of donor plants. Flower buds (at least 10 buds for each treatment) were surface-sterilized with sodium hypochlorite solution 3.5% (w/v) for 15 min. They were rinsed twice with sterile distilled water for 5 min and then they were blended in a washing solution (130 g sucrose in 1 l of distilled water) and filtered through a 63-μm nylon mesh. The crude microspore suspension was centrifuged twice at 1300 rpm for 5 min at 4°C (Beckman, Fullerton, USA). The freshly isolated microspores were dispensed in 1.5 ml Eppendorf tubes containing NLN medium with 13% sucrose (Lichter 1982) and at this stage, 2,4-D was applied at 4 concentrations (15, 25, 35 and 45 mg/l). After 15, 30 and 45 min, Eppendorf tubes were centrifuged (Heraeus, Osterode, Germany) at 2000 rpm for 2 min at 4°C and then 2,4-D treated microspores were washed twice with NLN medium without 2,4-D via centrifugation (2000 rpm for 2 min). Then the remaining suspension of microspores was diluted in 1.5 ml NLN medium containing 13% sucrose (Lichter 1982) and dispensed in multi-dishes (3/6–4 × 10^4 cells/ml in each well). Heat shock, in Topas: 32.5°C for 24 h (as described in Telmer *et al.* 1993) and in Hyola 420: 30°C for 14 days (Oroojloo *et al.* 2011) was applied to the microspores as the control to be able to evaluate the novel 2,4-D stress. After stress treatments (heat or 2,4-D), cultures were incubated at 25°C in dark conditions. After 3 weeks, embryos were visible with naked eye. After 4 weeks, embryos were transferred to the solid B5 medium (Gamborg *et al.* 1968) containing 0.3% Gelrite supplemented with GA3 (0.01 mg/l) and incubated in the dark conditions at 4°C for 10 days and then transferred to light conditions (16/8 h photoperiod) at 20°C for further regeneration. Normal well-developed plantlets were transferred to autoclaved soil for growth and development.

For cytological analysis (microspore vitality, microspore developmental stage and nuclear divisions), microspores cultured at various concentrations of 2,4-D were collected by centrifugation, stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under a Nikon (E100 m) fluorescence microscope, equipped with a Nikon digital dxm1200 camera (Nikon, Tokyo, Japan).

At least three replications for each treatment (10 buds for each treatment) were established. The frequency of embryos in different stages of embryogenesis, i.e. globular-shape, heart-shape and torpedo-shape ones, were counted. The number of plantlets with shoot or root as well as normal (well-developed) ones was also counted. Analysis of variance (ANOVA) was performed on the data. Duncan's test was used for means separation. The regeneration frequency was calculated

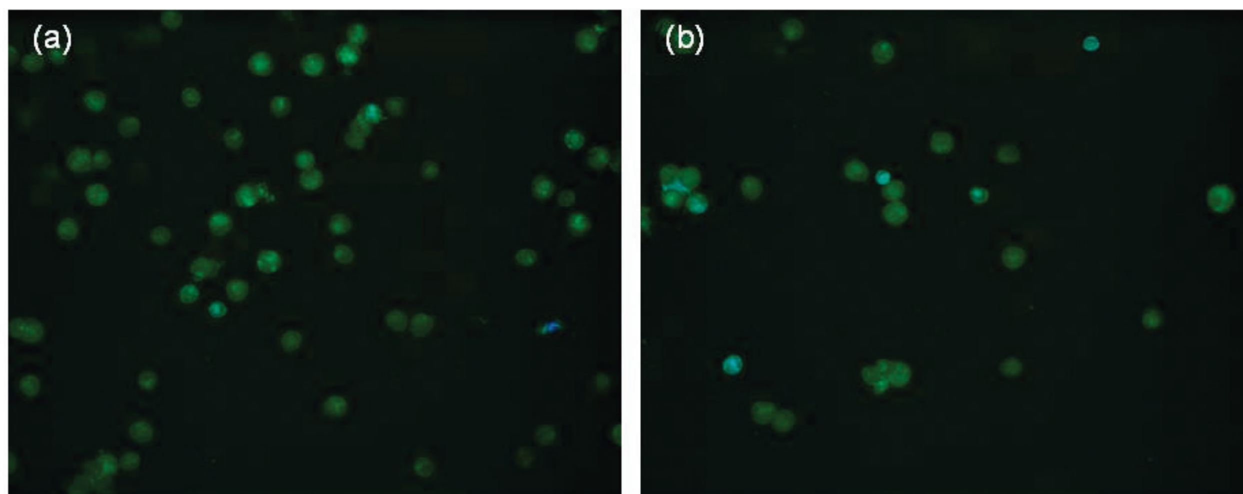

Figure 1. Viability of microspores stressed with 2,4-D (15 mg/l) and stained with FDA in *Brassica napus* cv. Topas; (a) 30 min, (b) 45 min

as the number of normal plantlets obtained from torpedo-stage embryos transferred to the regeneration medium.

RESULTS

Induction of embryogenesis and regeneration with 2,4-D

Microspores were subjected to 2,4-D at 15, 25, 35 and 45 mg/l for 15, 30 and 45 min. The treatment of microspores with 2,4-D for more than 45 min was extremely toxic and the viability of microspores decreased dramatically (Figure 1). The optimal duration of 2,4-D treatment to induce embryogenesis and keep viability in microspores was found to be 30 min (Figure 2). Isolated microspores kept at 25°C without applying heat or 2,4-D stress could not develop to embryos.

As shown in Figure 3, 2,4-D treatment at all concentrations applied to the microspores in *Brassica napus* cv. Topas at 25°C could induce embryogenesis and regeneration. The mean number of torpedo-embryos transferred to the regeneration medium was 13.3 in 2,4-D treatment at 15 mg/l for 30 min while in the control (32.5°C for 24 h) it was 113 (it means tenfold higher). The regeneration mean was the highest (7) when microspores were treated with 2,4-D at 25 mg/l for 30 min. However, the mean number of embryos and regenerants was higher in heat shock as compared to 2,4-D stress (Figure 3).

2,4-D stress could also induce microspore embryogenesis in *Brassica napus* cv. Hyola 420 (Figure 4). However, the frequency of embryos and regenerants

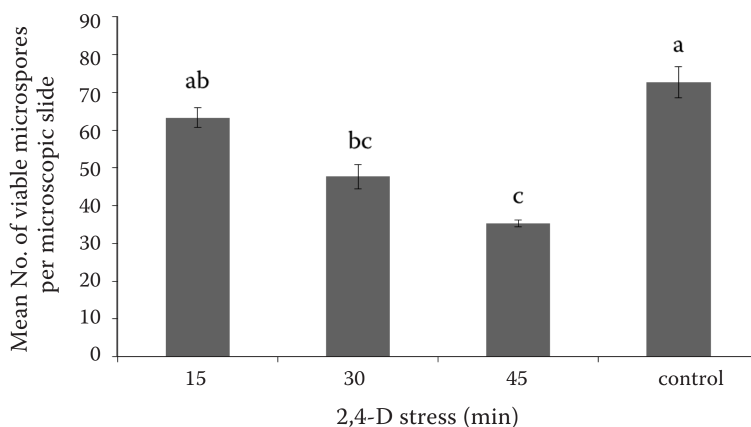

Figure 2. Viability of microspores stressed with 2,4-D (15 mg/l) for 15, 30, 45 min and of the control (without stress) in *Brassica napus* cv. Topas; means with different letters are significantly different at $P = 0.01$ (F-test)

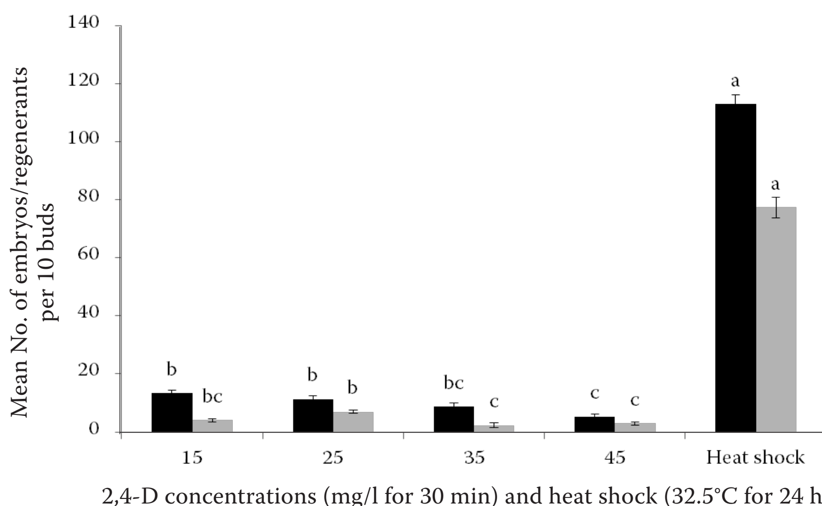

Figure 3. Mean number of embryos and regenerants per 10 buds in microspore culture of *Brassica napus* cv. Topas stressed with heat/2,4-D shock (black bar – embryos, gray bar – regenerants); means with different letters are significantly different at $P = 0.01$ (F-test)

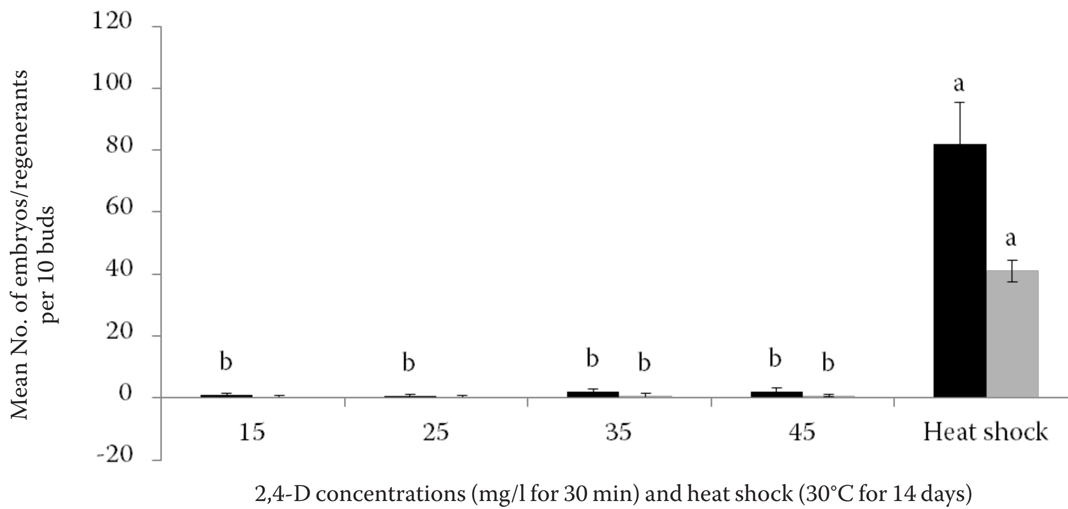

Figure 4. Mean number of embryos and regenerants per 10 buds in microspore culture of *Brassica napus* cv. Hyola 420 stressed with heat/2,4-D shock (black bar – embryos, gray bar – regenerants); means with different letters are significantly different at P = 0.01 (F-test)

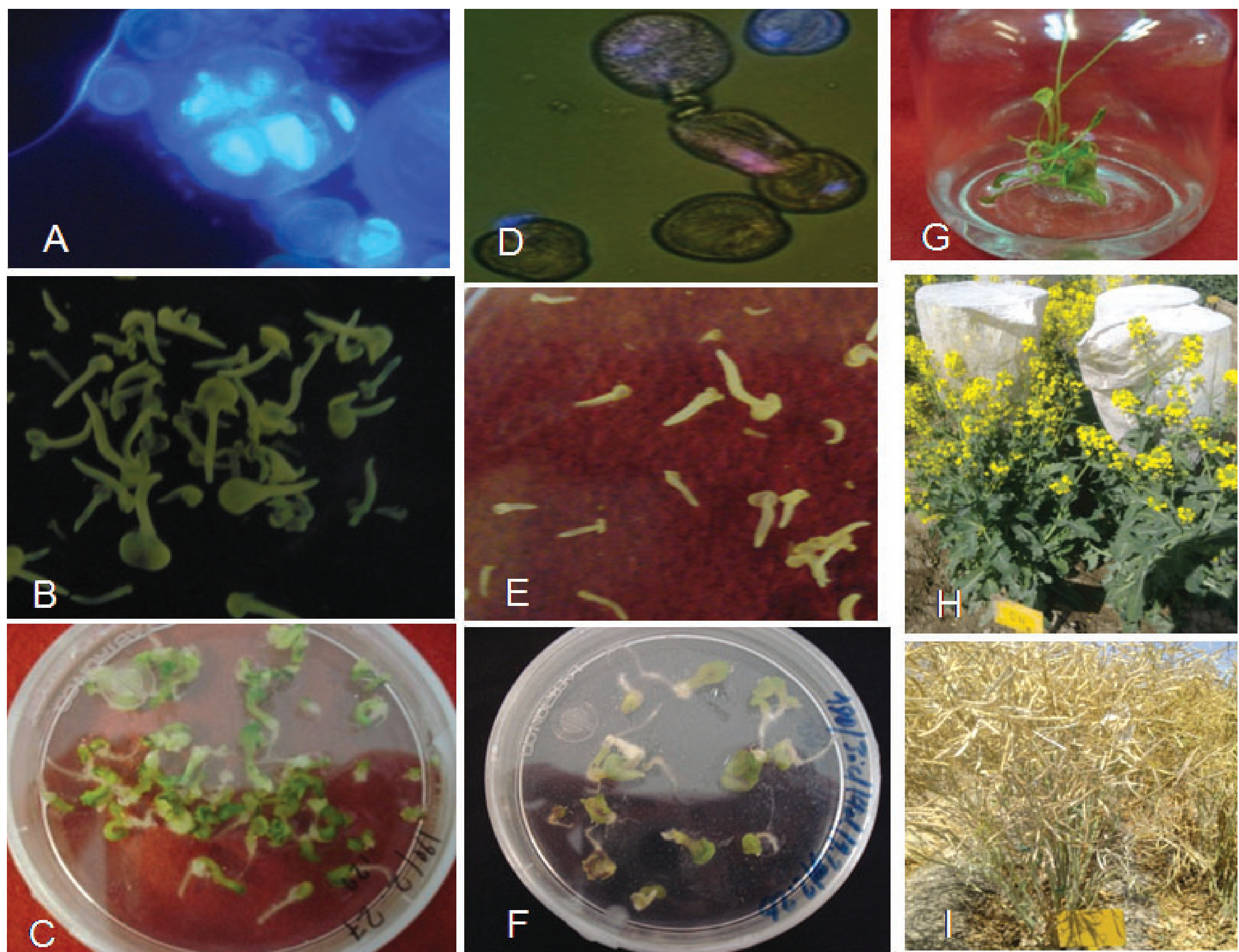

Figure 5. A, B, C – heat-induced multicellular structures and embryos; D, E, F – 2,4-D-induced multicellular structures and embryos; G – microspore-derived plantlet; H, I – DH lines

was lower as compared to the control (heat shock). In addition, more normal regeneration in Hyola 420 occurred at higher concentrations of 2,4-D (35 and 45 mg/l), however, the mean frequency was not significantly different (Figure 4). The quality of embryos in cultures induced with 2,4-D and washed after treatment was comparable to the heat-induced embryogenesis (Figure 5).

Developmental stage of microspores responsive to 2,4-D stress

The response of rapeseed microspores to 2,4-D treatment was dependent on the developmental stage of microspores. The most responsive stage was the late unicellular microspores similar to the heat shock and colchicine treatment (Figure 6).

Characterization of 2,4-D induced embryos

The frequency of suspensor-bearing embryos in microspores induced by 2,4-D was higher than in heat-induced microspores (Figure 7). Although the total number of embryos was higher in heat-induced embryogenesis, the ratio of suspensor-bearing embryos among the total embryos was significantly higher at all concentrations of 2,4-D as compared to the heat shock (Figure 7).

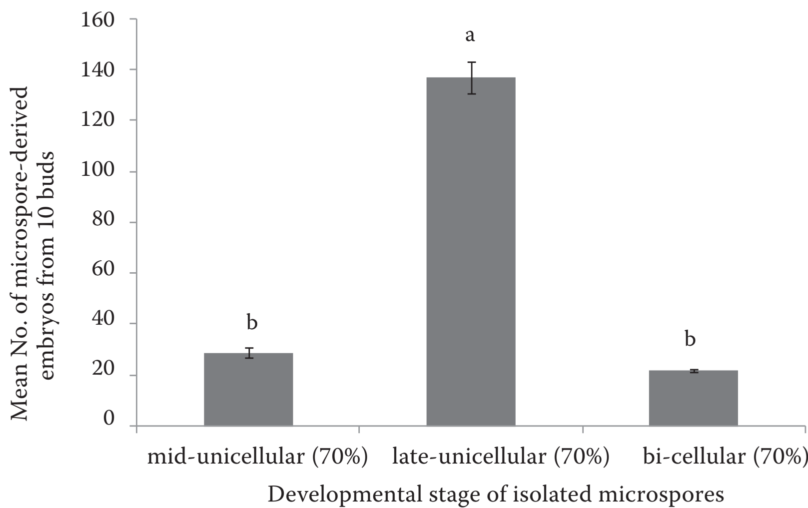

Figure 6. Mean number of embryos (per 10 buds) at different developmental stages of isolated microspores in *Brassica napus* cv. Topas; means with different letters are significantly different at $P = 0.05$ (F-test)

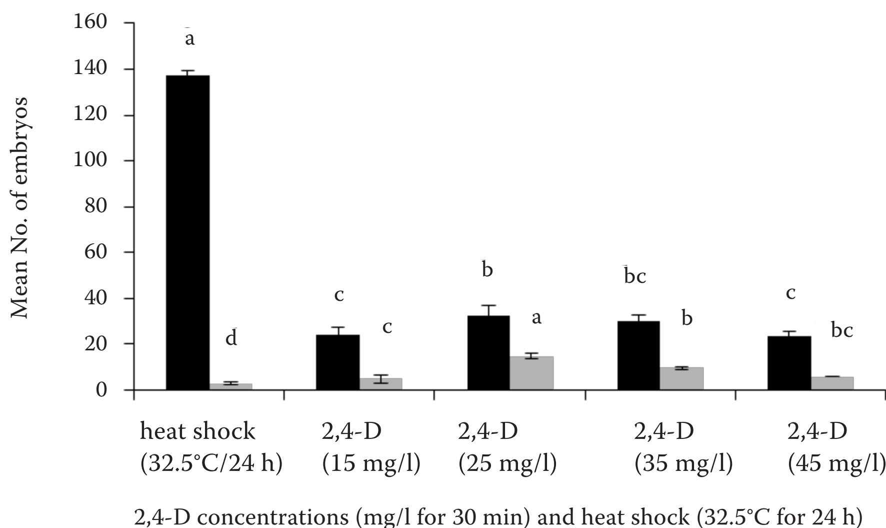

Figure 7. Mean number of total embryos and suspensor-bearing embryos (per 10 buds) in microspores of *Brassica napus* cv. Topas stressed with heat/2,4-D shock (black bar – total embryos, gray bar – suspensor-bearing embryos); means with different letters are significantly different at $P = 0.05$ (F-test)

DISCUSSION

2,4-D treatment at high concentrations for a short time (30 min) applied to the microspores in *Brassica napus* L. cvs. Topas and Hyola 420 at 25°C could induce embryogenesis and regeneration. In *Brassica napus* L. cv. Topas, microspores stressed with 2,4-D at all concentrations tested could produce embryos and regenerants. Although in *Brassica napus* L. cv. Hyola 420, more regenerants occurred at higher concentrations of 2,4-D (35 and 45 mg/l). Comparing the two cultivars tested, it is to note that the higher concentrations of 2,4-D should be applied in this genotype because a stronger heat shock is needed to induce embryogenesis in Hyola 420. Here we report the effect of 2,4-D applied alone as a novel stress for induction of embryogenesis in isolated microspores. Regarding the effect of 2,4-D as an inducer of embryogenesis, several mechanisms have been proposed as described below.

2,4-D causes different changes in physiology and gene expression of cells, implicating its possible role as a stress factor triggering the embryogenic pattern of development in cultured plant cells (Feher *et al.* 2003). Raghavan *et al.* (2006) evaluated the whole genome expression pattern of *Arabidopsis* in response to 2,4-D and indicated that 2,4-D not only modulated the expression of auxin, ethylene, and abscisic acid pathway but also regulated a wide variety of other cellular functions.

The effect of other induction treatments (cold, heat, starvation etc.) on the expression of genes such as heat shock protein (*Hsp*) genes in microspore embryogenesis has already been reported (for more details see Hosp *et al.* 2006). Recently, Hosp *et al.* (2011) reported that Nt *DCN1* gene encoding a 30-kDa protein comprising 259 amino acids is involved in cellular reprogramming and developmental transition of heat-stressed isolated tobacco microspores towards embryogenesis. They found that RNAi of DCN1 inhibited the stress-triggered reprogramming of cultured microspores from their intrinsic gametophytic mode of development to an embryogenic state. This stress-induced developmental switch is a known feature in many important crops and leads ultimately to the formation of haploid embryos and plants. They reported that one of the close homologues of Nt DCN1 in *Arabidopsis*, i.e. At3g28970 (ANTI-AUXIN-RESISTANT 3, *AAR3*) with 45% identity but without a UBA domain, has recently been shown to regulate root responses to 2,4-D (Bisvas *et al.* 2007). Therefore *NtDCN1* gene is activating the auxin cycle resulting in microspore embryogenesis induction. It could be proposed that 2,4-D might activate *NtDCN1* gene resulting in induction of embryogenesis. However, the hypothesis should be tested very carefully furthermore.

2,4-D stress can act as a modulator of somatic embryogenesis in plants via inducing the autonomous cell division (Cui *et al.* 1999; Pasternak *et al.* 2002). It has also been suggested that 2,4-D affects electrical patterns (Goldsworthy & Mina 1991), membrane permeability (Schauf *et al.* 1987) and IAA binding to the auxin-binding protein ABPI (Deshpande & Hall 2000).

If the induction of embryogenesis with 2,4-D is compared in somatic cells and microspores, it can be concluded that microspores need a shorter treatment of 2,4-D (less than one hour) in comparison with somatic cells (more than one day). It might be due to higher sensitivity of microspores to 2,4-D.

In general, it can be proposed that 2,4-D treatment at a high concentration for a short time might activate the sporophytic pathway of isolated microspores either directly via up-regulating the embryogenesis-inducing genes or indirectly through the expression of auxin, ethylene, and abscisic acid pathways which could affect the conversion of microspores towards embryogenesis.

As expected, late unicellular microspores were the most responsive stage to 2,4-D treatment similarly like to the heat shock and colchicine treatment (Zaki & Dickinson 1991; Custers *et al.* 1994; Zhao *et al.* 1996). In maize, in contrast, mid-unicellular microspores were the most responsive (Obert & Barnabas 2004).

2,4-D induced microspores produced more suspensor-bearing embryos in comparison with the heat shock. As shown by several research groups (Meinke 1991; Yeung & Meinke 1993), suspensors vary widely in size and morphology from a single cell to a massive column of several hundred cells. The suspensor provides nutrients and growth regulators for the embryo proper during the early stage of embryogenesis, after which the suspensor degenerates (Beers 1997; Schwartz *et al.* 1997; Wredle *et al.* 2001). In the heart-shaped stage of embryo, the activity of gibberellins (GA) in the suspensor is much higher than that in the embryo proper (Alpi *et al.* 1975). Therefore as described above, the suspensor plays an important role for embryo patterning and normal regeneration.

In conclusion, it should be noted that our achievement is the first report on the induction of mi-

crospore embryogenesis with 2,4-D treatment alone. We have also checked the 2,4-D effect as a stress to freshly isolated wheat microspores and according to the results obtained, 2,4-D could also induce embryogenesis (unpublished data). It is very important for plant species whose microspores are strongly sensitive to the classical stresses, i.e. heat, cold and carbon starvation. In such species, 2,4-D could be recommended for embryogenesis induction in microspores.

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