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ORIGINAL PAPER

Efficient induction of microspore embryogenesis using abscisic acid, jasmonic acid and salicylic acid in *Brassica napus* L

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Abstract The stress hormones abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) play an important role in the regulation of physiological processes and are often used in tissue culture to promote somatic embryogenesis and to enhance the quality of somatic embryos. Despite many studies on *Brassica napus* microspore culture, the effects of stress hormones (ABA, JA and SA) on microspore embryogenesis are not well explored. In this study, the effects of three incubation periods (6, 12 and 24 h) at different levels of ABA, JA and SA (0, 0.2, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}) on microspore embryogenesis of rapeseed (*B. napus* L.) cv. 'Regent' were investigated. ABA (0.5 mg l^{-1} for 12 h) enhanced microspore embryogenesis by about threefold compared with untreated cultures and increased normal plantlet regeneration by 68 %. ABA treatment also effectively reduced secondary embryo formation at all concentrations tested but enhanced callusing at high levels, for example 67 % at 1.0 mg l^{-1} for 24 h. Highest embryo yield (286.0 embryos Petri dish^{-1}) was achieved using 1.0 mg l^{-1} JA for 24 h and highest normal plantlet regeneration (54 %) was observed in cultures exposed to 0.5 mg l^{-1} JA for 12 h. JA (5.0 mg l^{-1} for 24 h) also reduced the germination of microspore-derived embryos on regeneration medium by 21 %. SA at 0.2 and 0.5 mg l^{-1} for 6 h increased microspore embryogenesis (184.0 and 193.4 embryos Petri dish^{-1}) relative to the control (136.2 embryos Petri dish^{-1}). However, SA did not improve normal regeneration, secondary embryo formation or callusing. Microspore embryogenesis and plant regeneration could be improved by ABA, JA as well as SA when the appropriate level and duration of incubation were selected.

Keywords Abscisic acid · Jasmonic acid · Salicylic acid · Callogenesis · Secondary embryogenesis

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Abbreviations

ABA	Abscisic acid
AOC	*Allene oxide cyclase*
AOS	*Allene oxide synthase*
ASA	Acetylsalicylic acid
GA_3	Gibberellic acid
JA	Jasmonic acid
LEA	Late embryogenesis abundant
Lox	*13-Lipoxygenase*
MDE	Microspore-derived embryo
MeJA	Methyl jasmonate
NO	Nitric oxide
PCD	Programmed cell death
PLB	Protocorm like body
ROS	Reactive oxygen species
SA	Salicylic acid

Introduction

Isolated microspore culture is likely to remain a prominent method in plant breeding as it allows for the rapid

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production of homozygous plants while, in the context of developmental biology, it allows for in vitro embryogenesis to be explored in greater detail. Microspore culture is also useful for gene transfer, for biochemical and physiological studies and for the production of desired traits such as herbicide resistance, fatty acid modification through mutagenesis and selection and also routinely used for establishing genetic mapping (Barro et al. 2002; Liu et al. 2005; Abdollahi et al. 2009; Santra et al. 2012). However, all of these applications depend heavily on the efficiency of microspore embryogenesis.

Microspore embryogenesis is a process in which the gametophytic pollen program of the microspore is reoriented towards a new embryo sporophytic program, a process that requires stress treatment, usually performed in the anther or isolated microspores (Shariatpanahi et al. 2006; Jacquard et al. 2009; Ferrie and Caswell 2011). Several abiotic stresses, alone or in combination, are used to trigger microspore embryogenesis. In the genus *Brassica*, microspores are usually inducted by osmotic stress (Ferrie et al. 1999; Ferrie and Keller 2007), heat shock pretreatment (Ferrie et al. 1999; Prem et al. 2005) and its duration (Ahmadi et al. 2012a), mutagenic agents (Ahmadi et al. 2012b) or antioxidants such as ascorbic acid, alpha-tocopherol and glutathione (Hoseini et al. 2013). Microspores up-regulate abscisic acid (ABA) biosynthesis following an inductive stress (Reynolds and Crawford 1996). Interestingly, ABA is behind some genes that are up-regulated during the embryogenesis induction phase and a large number of ABA-regulated genes involved in the induction of embryogenesis have been cloned and studied. One of these is a wheat (*Triticum aestivum* L.) gene encoding an ABA-inducible, embryo-specific cycteinlabeled class II metallothionein protein (Reynolds 2000). Another is a barley (*Hordeum vulgare* L.) gene coding for an ABA-regulated alcohol dehydrogenase (Maraschin et al. 2006). The internalization of stress signals by the ABA signaling pathway would be an intermediate step in the activation of gene expression programs leading, either directly or indirectly, to microspore embryogenesis (Maraschin et al. 2005; Tsuwamoto et al. 2007).

Jasmonic acid (JA) is a lipid-based hormone signal which not only regulates a wide range of processes in plants, ranging from growth and photosynthesis to reproductive development but also is critical for plant defense against biotic and abiotic stresses (He et al. 2002). In comparison with ABA, information about the regulatory role of JA in different aspects of somatic embryogenesis is rather limited. JA inhibits somatic embryo formation in *Medicago sativa* (Ruduś et al. 2001) but Teixeira da Silva (2013) found that JA improved protocorm-like body (PLB) formation, which is synonymous to somatic embryogenesis in orchids. Working on *H. vulgare* anther cultures, Jacquard et al. (2009) noted a higher expression of three genes *13-Lipoxygenase*), *Allene oxide cyclase* and *Allene oxide synthase* encoding enzymes involved in the first step of JA biosynthesis in embryogenic anthers of *H. vulgare* after low temperature (4 °C in the dark for 3–96 h) and osmotic stress (62 g l^{-1} mannitol for providing osmotic pressure) in comparison to non-embryogenic cultures.

Salicylic acid (SA), a plant phenolic derivative is now considered to be a hormone-like endogenous regulator and its role in the defense mechanisms against biotic and abiotic stress is well documented (Catinot et al. 2008). Besides its function during biotic and abiotic stress, SA plays a crucial role in the regulation of physiological and biochemical processes during the entire lifespan of plant. Being a mobile molecule, SA is capable of acting as a cell signal that senses, amplifies, and transmits information from a cell and might help in programming embryogenesis (Mulgund et al. 2012). Several reports describe exogenous SA and its acetylated form, acetylsalicylic acid, in enhanced somatic embryogenesis in carrot (*Daucus carota*), geranium (*Pelargonium* × *hortorum* Bailey), *Astragalus adsurgens* Pall and *Pinus roxburghii* (Hutchinson and Saxena 1996; Luo et al. 2001; Hao et al. 2006; Malabadi et al. 2008). SA stimulates somatic embryogenesis by regulating cell division and enlargement, and activates DNA replication without concomitant nuclear division (Luo et al. 2001). In this study, the effects of different levels and durations of exposure to ABA, JA and SA on the efficiency of microspore embryogenesis and subsequently, the regeneration of microspore-derived embryos (MDEs) were assessed to improve microspore embryogenesis and plantlet regeneration in *Brassica napus*.

Materials and methods

Donor plants and growth conditions

B. napus cv. 'Regent' was the test plant. Seeds were kindly provided by the Dryland Agriculture Research Institute (Maragheh) of Iran. Donor plants were grown in a growth chamber at a day/night temperature of 15/10 °C with a 16-h photosynthetic photon flux density (PPFD) of 400 μE m^{-2} s^{-1}. Plants were irrigated three times a week.

Microspore culture

Microspore culture was carried out according to Coventry and Kott (1998) with some modifications. Buds 2–3 mm in length containing a mixed population of mid to late-uninucleate microspores were harvested from the main and lateral branches of donor plants that had reached anthesis

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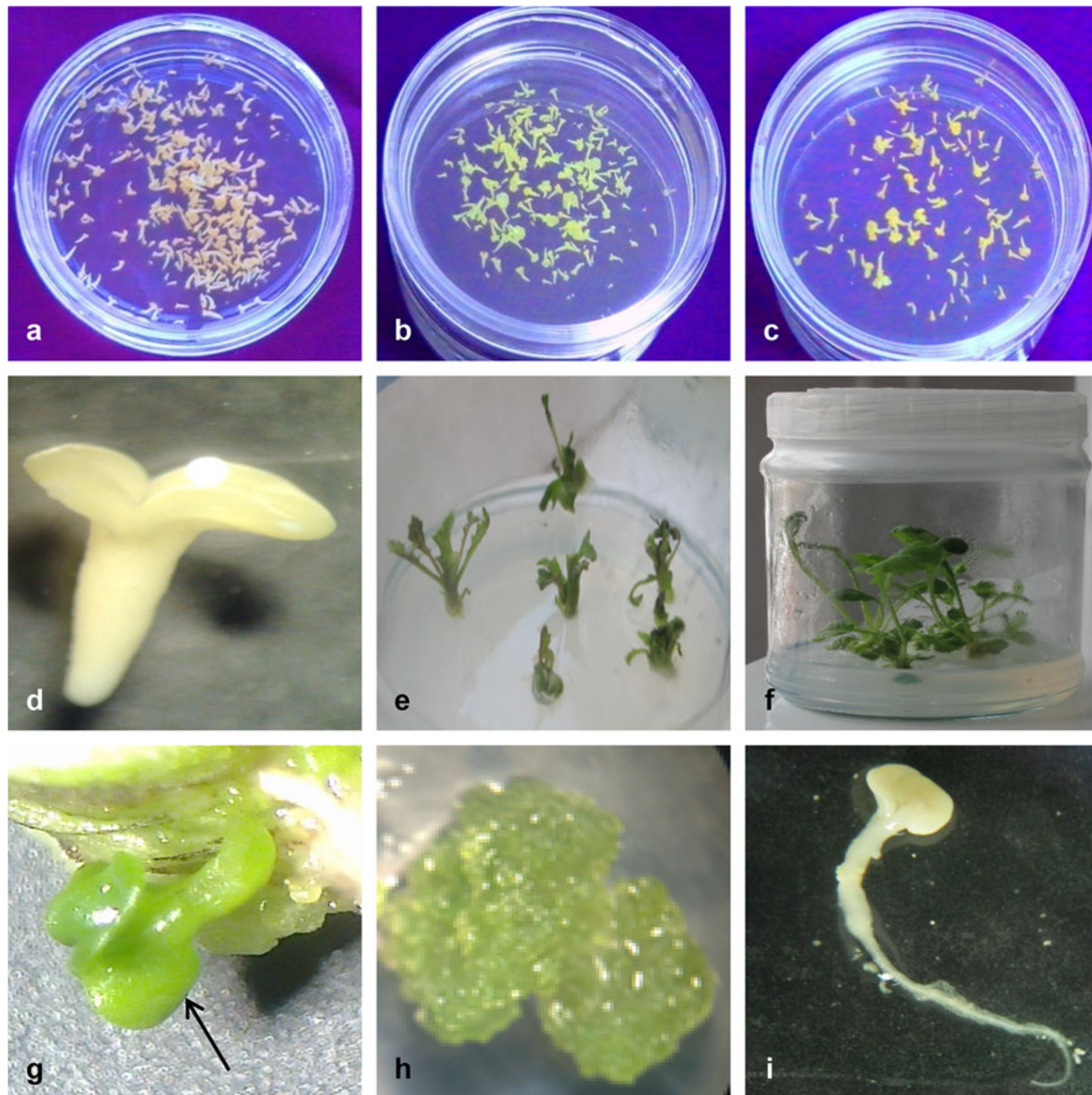

Fig. 1 *B. napus* L. cv. 'Regent' microspore embryogenesis in the presence of **a** ABA (0.5 mg l^{-1} for 12 h); **b** JA (1.0 mg l^{-1} for 24 h); **c** SA (1.0 mg l^{-1} for 6 h); **d** cotyledonary MDE (3 weeks after microspore culture); **e**, **f** normal plantlet regeneration (5 and 7 weeks after microspore culture); **g** secondary embryo formation (0.2 mg l^{-1} ABA for 24 h, showed by arrow); **h** callogenesis (5.0 mg l^{-1} ABA for 6 h); **i** rooted MDE in original NLN-13 medium (5.0 mg l^{-1} SA for 6 h)

after about 80–100 days. These buds were immersed in 5.25 % sodium hypochlorite (Golrang, Tehran, Iran) with gentle shaking for 10 min followed by two 5-min washes with cold (4 °C) sterile distilled water. Approximately 70–80 sterilized buds were placed in a glass tube and were gently macerated into 15 ml of liquid NLN-13 (Lichter 1982) medium supplemented with 13 % (w/v) sucrose (Merck, Darmstadt, Germany) using a sterile glass rod. The crude suspension was filtered through a 52 µm metal mesh (Damavand Tes Sieve Ltd. Tehran, Iran), collected into two 50-ml centrifuge tubes and the volume was adjusted with fresh NLN-13 medium to 25 ml. The filtrate was centrifuged at $100\times g$ for 5 min with an EBA 20, Behsan (Tehran, Iran) centrifuge. The supernatant was decanted and the pellet was rinsed in fresh NLN-13 medium. This procedure was repeated twice. Finally, plating density was adjusted to 2×10^4 microspores ml^{-1} using a hemocytometer (Precicolor, Germany). The microspore suspension (5 ml) was dispensed into 6-cm sterile plastic Petri dishes (Farazbin, Tehran, Iran) and cultures were incubated at 30 ± 0.5 °C in the dark for 12 days and then transferred to 24 ± 0.5 °C. Once embryos were visible to the naked eye (Fig. 1a–c), the Petri dishes were transferred onto a rotary shaker in the dark at 55 rpm.

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Table 1 Mean number of embryos formed Petri dish^{-1} for various levels of ABA and duration of incubation in *B. napus* L. cv. 'Regent'

ABA level (mg l^{-1})	Duration of ABA treatment		
	6 h	12 h	24 h
0	134.0 ± 14.1 c*	129.2 ± 13.6 d	137.8 ± 15.6 b
0.2	171.2 ± 15.2 b	268.0 ± 16.3 b	194.0 ± 12.9 a
0.5	212.2 ± 16.7 a	391.4 ± 18.1 a	143.0 ± 14.2 b
1.0	123.8 ± 15.4 c	233.0 ± 14.5 c	64.4 ± 11.4 c
2.0	38.4 ± 12.6 d	25.2 ± 11.8 e	0.0 d
5.0	19.6 ± 10.7 e	0.0 f	0.0 d

* Within a column, means (± SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

ABA, JA, and SA treatment

ABA and JA (Sigma-Aldrich, St. Louis, MO, USA) as well as SA (Duchefa Biochemie, Haarlem, The Netherlands) were dissolved in ethanol (99 %) in the dark using gentle shaking at room temperature. The pH was adjusted to 6.0 with 1 N NaOH and 1 N HCL and maintained in a refrigerator at −20 °C until needed. After determining plating density (2×10^4 microspores ml^{-1}) and dispensing microspore suspension into Petri dishes, different levels of ABA, JA and SA (0, 0.2, 0.5, 1.0, 2.0 and 5.0 mg l^{-1}) were added to the culture medium for three time periods (6, 12 and 24 h) and incubated at 30 °C in dark. Hormone residuals were removed by centrifugation at $150\times g$ for 5 min. Plating density was adjusted to 2×10^4 microspores ml^{-1} and the suspension was dispensed into the same Petri dishes and incubated at 30 °C.

MDE regeneration

Cotyledonary MDEs (Fig. 1d) measuring 4–6 mm in length were transferred onto B5 medium (Gamborg et al., 1968) containing 0.1 mg l^{-1} gibberellic acid (GA_3, Fluka, Buchs, Switzerland), 2 % sucrose, pH 5.7 and 0.7 % agar (Duchefa Biochemie). After incubating at 4 ± 0.5 °C in the dark for 1 week, the Petri dishes were maintained at 25 ± 1 °C under a 16-h photoperiod with a PPFD of 40 µE m^{-2} s^{-1} for 1 week. Subsequently, the distal half of the cotyledons was sliced off and the MDEs were placed onto B5 medium (Gamborg et al. 1968) containing 1 % sucrose, pH 5.7, 0.9 % agar and without GA_3 for plantlet regeneration (Fig. 1e, f, Ahmadi et al. 2012b).

Experimental design and statistical analysis

The experiments were conducted in a factorial arrangement based on completely randomized design to evaluate the effect of different factors. Each treatment had five replications (Petri dishes). Entire experiments were repeated twice. Data analyses were performed using SPSS software version 17. The means were compared using Duncan's multiple range (DMRT) test at $\alpha = 0.01$ following analysis of variance and frequencies (%) were compared using a Chi square test at $\alpha = 0.01$.

Table 2 Effect of various levels of ABA and duration of incubation on frequency (%) of production of whitish MDEs following pretreatment at 4 °C for 1 week in *B. napus* L. cv. 'Regent' (for each treatment, 100 MDEs were randomly counted)

ABA level (mg l^{-1})	Duration of ABA treatment		
	6 h	12 h	24 h
0	35	42	39
0.2	24	21	15
0.5	16	8	13
1.0	7	11	6
2.0	4	9	0
5.0	5	–	–

Within a column, no significant differences were found according to the chi-squared test ($P = 0.01$)

Results

ABA treatment

ABA level and duration of exposure significantly affected microspore embryogenesis (Table 1). Embryo production was increased about threefold in cultures exposed to 0.5 mg l^{-1} ABA for 12 h in contrast to untreated cultures. However, there was a sharp decrease in microspore embryogenesis as ABA levels increased with 5.0 mg l^{-1} ABA completely inhibiting microspore embryogenesis in cultures incubated for 12 and 24 h. Following incubation of MDEs at 4 °C for 1 week, many infected or fully undeveloped MDEs turned white and therefore decanted from the Petri dishes. However, ABA treatment reduced the number of whitish MDEs (Table 2). No MDEs turned white when cultures were exposed to 2.0 mg l^{-1} ABA for 24 h. ABA

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Table 3 Effect of various levels of ABA and duration of incubation on three paths of MDE regeneration (normal regeneration, secondary embryogenesis and callogenesis) in *B. napus* L. cv. 'Regent' (for each treatment, 100 MDEs were randomly counted)

Duration of ABA treatment	ABA level (mg l^{-1})	Type of MDE regeneration (%)		
		Normal regeneration	Secondary embryogenesis	Callogenesis
6 h	0	22*	38	40
	0.2	33	24	43
	0.5	54	11	35
	1.0	45	19	36
	2.0	28	15	57
	5.0	19	14	67
12 h	0	20	44	36
	0.2	41	9	50
	0.5	68	5	32
	1.0	48	9	43
	2.0	24	11	65
	5.0	–	–	–
24 h	0	24	35	41
	0.2	37	16	47
	0.5	49	11	40
	1.0	21	12	67
	2.0	–	–	–
	5.0	–	–	–

* Within a column, no significant differences were found according to the chi-squared test ($P = 0.01$)

Table 4 Mean number of embryos formed Petri dish^{-1} for various levels of JA and duration of incubation in *B. napus* L. cv. 'Regent'

JA level (mg l^{-1})	Duration of JA treatment		
	6 h	12 h	24 h
0	126.2 ± 16.1 b*	136.6 ± 13.3 c	131.0 ± 12.8 d
0.2	133.0 ± 12.3 b	132.4 ± 11.8 c	157.4 ± 14.2 c
0.5	129.8 ± 14.5 b	145.2 ± 16.2 c	211.2 ± 17.9 b
1.0	134.2 ± 12.8 b	196.8 ± 13.7 b	286.0 ± 17.1 a
2.0	208.4 ± 17.6 a	254.0 ± 18.7 a	168.8 ± 14.2 c
5.0	217.6 ± 16.4 a	107.4 ± 15.5 d	46.2 ± 10.3 e

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

treatment also affected the path of MDE regeneration (via normal regeneration, secondary embryogenesis or callogenesis) into whole plantlets (Table 3). The highest normal regeneration (68 %) was observed with 12 h treatment of 0.5 mg l^{-1} ABA. ABA treatment also effectively reduced secondary embryo formation (Fig. 1g) at all levels tested but enhanced callusing (Fig. 1h) at high levels e.g. 67 % at 5.0 mg l^{-1} for 6 h. ABA (0.5 mg l^{-1}) for 12 h was the optimum combination for improving the production of MDEs and for subsequent normal plantlet regeneration.

Table 5 Effect of various levels of JA and duration of incubation on germination (%) of the MDEs in *B. napus* L. cv. 'Regent' (for each treatment, 100 MDEs were randomly counted)

JA level (mg l^{-1})	Duration of JA treatment		
	6 h	12 h	24 h
0	96	89	94
0.2	78	74	83
0.5	81	76	78
1.0	73	69	62
2.0	55	49	42
5.0	34	28	21

Within a column, no significant differences were found according to the chi-squared test ($P = 0.01$)

Table 6 Effect of various levels of JA and duration of incubation on three paths of MDE regeneration (normal regeneration, secondary embryogenesis and callogenesis) in *B. napus* L. cv. 'Regent' (for each treatment, 100 MDEs were randomly counted)

Duration of ABA treatment	ABA level (mg l^{-1})	Type of MDE regeneration (%)		
		Normal regeneration	Secondary embryogenesis	Callogenesis
6 h	0	26*	35	39
	0.2	33	38	29
	0.5	29	34	27
	1.0	31	38	31
	2.0	24	31	45
	5.0	21	27	52
12 h	0	23	38	39
	0.2	36	29	35
	0.5	54	24	22
	1.0	29	34	37
	2.0	22	25	53
	5.0	18	21	61
24 h	0	21	36	43
	0.2	31	27	42
	0.5	28	24	48
	1.0	24	21	55
	2.0	18	17	65
	5.0	12	15	73

* Within a column, no significant differences were found according to the chi-squared test ($P = 0.01$)

JA treatment

Highest embryo yield (286.0 embryos Petri dish^{-1}) was possible with 1.0 mg l^{-1} JA for 24 h (Table 4). Although there was no significant difference among low levels of JA (0, 0.2, 0.5 and 1.0 mg l^{-1}), higher levels (2.0 and 5.0 mg l^{-1}) resulted in significantly higher embryogenesis when cultures were incubated for 6 h. However, at longer incubations,

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Table 7 Mean number of embryos formed Petri dish^{-1} for various levels of SA and duration of incubation in *B. napus* L. cv. 'Regent'

ABA level (mg l^{-1})	Duration of ABA treatment		
	6 h	12 h	24 h
0	136.2 ± 16.2 b*	131.0 ± 14.8 a	127.8 ± 16.6 a
0.2	193.4 ± 19.4 a	112.4 ± 16.6 b	97.2 ± 13.5 b
0.5	184.0 ± 14.3 a	88.0 ± 13.2 c	69.8 ± 15.8 c
1.0	139.8 ± 15.9 b	54.2 ± 9.7 d	28.0 ± 9.8 d
2.0	97.2 ± 10.7 c	0 e	0 e
5.0	43.0 ± 10.2 d	0 e	0 e

* Within a column, means (±SD) followed by the same letters are not significantly different according to DMRT ($P = 0.01$)

Table 8 Effect of various levels of SA and duration of incubation on three types of MDE regeneration (normal regeneration, secondary embryogenesis and callogenesis) in *B. napus* L. cv. 'Regent' (for each treatment, 100 MDEs were randomly counted)

Duration of ABA treatment	ABA level (mg l^{-1})	Type of MDE regeneration (%)		
		Normal regeneration	Secondary embryogenesis	Callogenesis
6 h	0	33*	29	38
	0.2	35	32	33
	0.5	31	34	35
	1.0	33	36	31
	2.0	27	32	41
	5.0	21	28	51
12 h	0	37	27	36
	0.2	33	35	32
	0.5	36	28	36
	1.0	29	33	38
	2.0	–	–	–
	5.0	–	–	–
24 h	0	29	31	40
	0.2	31	34	35
	0.5	26	36	38
	1.0	23	34	43
	2.0	–	–	–
	5.0	–	–	–

* Within a column, no significant differences were found according to the chi-squared test ($P = 0.01$)

namely 12 and 24 h with 5.0 mg l^{-1} JA, microspore embryogenesis and MDE germination on regeneration medium decreased (e.g. 21 %, Table 5). JA levels and the duration of its application also affected the regeneration of MDEs. Highest normal plantlet regeneration (54 %, Table 6) was achieved in cultures exposed to 0.5 mg l^{-1} JA for 12 h. High levels of JA (2.0 and 5.0 mg l^{-1}) resulted in higher callusing at all durations tested.

SA treatment

SA treatment (0.2 and 0.5 mg l^{-1}) during the 6-h exposure caused a significant increase in the number of MDEs generated Petri dish^{-1} (Table 7). However, SA treatment at longer durations of incubation decreased microspore embryogenesis, with 2.0 and 5.0 mg l^{-1} SA for 12 and 24 h completely inhibiting microspore embryogenesis. However, initial cell divisions were observed, but these failed to proceed further into fully developed MDEs. In addition, few embryos rooted (Fig. 1i) in cultures exposed to 5.0 mg l^{-1} SA for 6 h in NLN-13 medium (4–9 rooted MDEs Petri dish^{-1}). SA did not improve normal regeneration nor did it affect secondary embryo formation and callusing (Table 8).

Discussion

ABA is produced by plants in response to a number of stressors, including some that are used to induce androgenesis. Our results revealed that microspore embryogenesis is strongly affected by ABA treatment. 12 h treatment of 0.5 mg l^{-1} ABA had the best effect on embryogenesis (391.4 embryos Petri dish^{-1}). Comparative studies between two cultivars of barley ('Digger' and 'Igri') revealed that recalcitrance to androgenesis was due to differences in the endogenous levels of ABA and a decrease in the number of viable microspores in 'Digger'. In contrast, 'Igri' was responsive to ABA and produced more viable microspores upon pre-treatment with mannitol and showed increased regeneration efficiency (Van Bergen et al. 1999). ABA influences the viability of microspores by preventing programmed cell death (PCD) and stimulates androgenesis (Van Bergen et al. 1999; Wang et al. 1999). PCD, also known as apoptosis, is a physiological cell death process in which a cell guides its own destruction following biotic or abiotic stresses (Wang et al. 1999; Carimi et al. 2003). Inductive stresses produce reactive oxygen species and nitric oxide signaling molecules that mediate cellular responses and PCD, modifying the embryogenic microspore response and therefore, the efficiency of the process (Rodríguez-Serrano et al. 2012). In addition, Zur et al. (2008) reported that increased levels of ABA in anther culture of triticale was associated with the higher microspore culture viability and thus increased embryogenesis induction. Akula et al. (2000) investigated the role of various levels of ABA in addition to osmotic compounds on the induction of somatic embryos in tea. They found that induction was enhanced when 7.5 mg l^{-1} ABA was co-supplemented with betaine in the induction medium. However, in our experiment, high levels of ABA (2.0 and 5.0 mg l^{-1}) significantly reduced microspore

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embryogenesis. In order to regenerate plantlets, 4–6 mm MDEs were subjected to 4 °C for 1 week in the dark. According to Zhang et al. (2006), exposing MDEs to chilling promotes normal regeneration (Table 2), but in this study many MDEs turned white when incubated in the cold. ABA treatment decreased the number of whitish MDEs in contrast to untreated cultures. The exogenous application of ABA is associated with protein synthesis and compatible solutes, which play an important role in enhancing freezing tolerance (Fang et al. 2004).

When MDEs are placed on regeneration medium, the cotyledons turn green, a primary root develops, but the plumule rarely produces a shoot and thus many MDEs do not regenerate normally (Kott and Beversdorf 1990; Babbar et al. 2004). The majority of implanted MDEs on regeneration medium develop into an irregular mass of tissue, i.e., callogenesis (Fig. 1h) or regenerate via secondary embryo formation on epidermal cells of either the hypocotyl or cotyledons (Fig. 1g; Kott and Beversdorf 1990; Babbar et al. 2004; Ahmadi et al. 2012b). ABA not only affected microspore embryogenesis but also the type of regeneration (via normal regeneration, secondary embryo formation or callogenesis) of the MDEs into whole plantlets. The highest normal plantlet regeneration was achieved with 0.5 mg l^{-1} ABA at all the durations tested. A number of factors limit the conversion of somatic embryos into normal plantlets, the most important of which are considered to be a poor quality and incomplete maturation of somatic embryos (Choudhary et al. 2009; Rai et al. 2009). During somatic embryo maturation, several storage and late embryogenesis abundant (LEA) proteins, fatty acid reserves and sugars, all required for normal germination, are synthesized, and mature somatic embryos which have accumulated enough storage materials, develop into normal plantlets (von Arnold et al. 2002; Sharma et al. 2004). It is now well understood that both the synthesis and deposition of these storage and LEA proteins are regulated by ABA (von Arnold et al. 2002; Ghelis et al. 2008). ABA treatment reduced the percentage of secondary embryo formation at all levels tested but higher level, namely 2 and 5 mg l^{-1} for 6 h resulted in higher callogenesis (Table 3).

JA level and duration also affected microspore embryogenesis and the regeneration of derived embryos and 1.0 mg l^{-1} JA for 24 h was the best combination for microspore embryogenesis. Working on saffron somatic embryogenesis, Blázquez et al. (2004) found that 0.5 mg l^{-1} JA significantly improved somatic embryogenesis. Jasmonates are a widely disturbed group of plant growth regulators in the plant kingdom and JA owes its growth-promotive activity due to an increase in cell division and expansion (Takahashi et al. 1994). However, high levels i.e., 5.0 mg l^{-1} JA at 12 and 24 h reduced microspore embryogenesis and produced pale-yellow embryos many of which failed to proceed further into fully developed plantlets (data not shown). According to Teixeira da Silva (2012), 1.0 mg l^{-1} methyl jasmonate (MeJA) improved PLB (syn. somatic embryo) formation in hybrid *Cymbidium*, but PLB formation decreased as MeJA level increased. Our results also revealed that high levels of SA (2.0 and 5.0 mg l^{-1}), when incubated for 24 h, reduced the germination of MDEs. JA and MeJA inhibit embryo germination in angiosperms (Preston et al. 2002; Bialecka and Kepczynsky 2003). One reason for this is related to the inhibition of ethylene production which is required for embryo germination (Nojavan-Asghari and Ishizava 1998).

SA (2.0 and 0.5 mg l^{-1} for 6 h) could also improve microspore embryogenesis relative to untreated cultures. Sakhanokho et al. (2009) noted that the addition of 14 mg l^{-1} SA improved *Hedychium bousigonianum* somatic embryogenesis, but was reduced at 21 mg l^{-1} SA, suggesting an inhibitory effect of high SA levels on somatic embryogenesis. However, even at this level, somatic embryo production was twice that of untreated cultures (Sakhanokho et al. 2009). The inhibitory effect of high levels of SA on somatic embryogenesis has been reported in other crops. Roustan et al. (1989) found that 7 mg l^{-1} SA was optimal for somatic embryo production in carrot suspension cultures but high levels (35 mg l^{-1}) were toxic. Luo et al. (2001) reported a decrease in somatic embryogenesis at levels exceeding 24 mg l^{-1} in *A. adsurgens* Pall. However, Sakhanokho et al. (2009) did not observe any toxic effect or necrosis at high levels (14 and 21 mg l^{-1}) of SA. According to our results, 2.0 and 5.0 mg l^{-1} of SA, when incubated for 12 and 24 h, completely inhibited microspore embryogenesis. However, initial divisions were observed but these failed to proceed further into fully developed MDEs. The exact toxic level of SA may be genotype-dependant.

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