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ORIGINAL PAPER

# Disturbance of ethylene biosynthesis and perception during somatic embryogenesis in *Medicago sativa* L. reduces embryos' ability to regenerate

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**Abstract** Effects of non-specific ethylene biosynthesis inhibitors: salicylic acid (SA) and aminoethoxyvinylglycine (AVG), and of specific inhibitors of ethylene binding to receptors: 1-methylcyclopropene (1-MCP) and 2,5-norbornadiene (NBD) applied during proliferation and differentiation phases of indirect somatic embryogenesis (SE) of *Medicago sativa* L. cv. Rangelander on embryogenic suspension growth, embryo production, development, and ability to germinate and convert were studied. Application of SA and AVG alone or together at concentrations from 1 to 500 μM in $B_5$g liquid medium during the proliferation phase had an inhibitory effect on ethylene production and embryogenic suspension growth. Additionally, it caused a drastic reduction in production of embryos and their development on BOi2Y solid differentiation medium. The inhibitory effect of SA was more visible than that of AVG. In addition, disturbance of ethylene biosynthesis during the proliferation phase of SE resulted in diminished lateral germination and conversion of cotyledonary embryos on MS solid medium. Moreover, blocking of ethylene receptors by 1-MCP during the proliferation phase also inhibited ethylene production and embryogenic suspension growth and reduced embryo production during differentiation. MCP almost completely inhibited development of cotyledonary embryos. At the same time, development of more embryos was arrested at the globular stage, and the number of abnormal embryos almost doubled. Similarly, addition of 1-MCP or NBD to the ambient atmosphere during the differentiation phase evidently arrested the development of embryos and, consequently, their ability to germinate and convert on MS regeneration medium. All the results presented above demonstrated that not only ethylene biosynthesis, but also ethylene action is involved in the control of individual phases of SE in *Medicago sativa* L. cv. Rangelander. And what is more, disturbance of these processes during distinct phases of SE adversely affects vigor of the somatic embryos obtained.

**Keywords** Ethylene production · Inhibitors of ethylene biosynthesis (SA, AVG) and action (1-MCP, NBD) · Somatic embryogenesis · Embryos' germination and conversion

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| ACC | 1-Aminocyclopropane-1-carboxylic acid |
| ACO | ACC-oxidase |
| ACS | ACC-synthase |
| AOA | Aminooxyacetic acid |
| AOS | Allene oxide synthase |
| AVG | Aminoethoxyvinylglycine |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| DNP | 2,4-Dinitrophenol |
| JA | Jasmonic acid |
| 1-MCP | 1-Methylcyclopropene |
| MGBG | Methylglyoxal bis(guanylhydrazone) |
| NAA | α-Naphthaleneacetic acid |
| NBD | 2,5-Norbornadiene |
| PPFD | Photosynthetic photon flux density |
| SA | Salicylic acid |
| SE | Somatic embryogenesis |

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## Introduction

Ethylene, a gaseous plant hormone, has long been recognized as a growth inhibitor (Abeles et al. 1992), but evidence is accumulating that it can also promote growth (Pierik et al. 2006). In higher plants, ethylene is synthesized from methionine through *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC), the synthesis being catalyzed by ACC synthase (ACS) and ACC-oxidase (ACO), respectively (Yang and Hoffman 1984). Ethylene production depends on both the type of tissue and the stage of its development as well as on environmental factors. Many physiological and molecular studies have demonstrated that the synthesis and perception of ethylene is required for in vivo vegetative development (Smalle and Van Der Straeten 1997; Kępczyński and Kępczyńska 2005), senescence of leaves, flowers and fruits (Wang et al. 2002) and germination of some seeds (Kępczyński and Kępczyńska 1997; Matilla and Matilla–Vazquez 2008; Nascimento 2003). Knowledge on the effects of ethylene on the regulation of different physiological processes that occur during in vitro plant tissue and organ culture, particularly during zygotic and somatic embryogenesis, is still very scant (Matilla and Matilla–Vazquez 2008). During zygotic embryogenesis of *Brassica juncea* L. and *B. napus* L., ethylene production peaked at the torpedo stage of embryogenic development (Johnson-Flanagan and Spencer 1994), while in *Cicer arietinum*, amount of ACC, the activities of ACS and ACO as well as ethylene production reached a maxima at the mid-stage of embryogenesis, declining during late embryogenesis and seed desiccation (Gomez-Jimenez et al. 1998). In *Brassica rapa* L., Rodrigez–Gacio et al. (2004) demonstrated that transcript *BR ACO1* (one cDNA clone coding for an ACC-oxidase) was accumulated only at the earliest phases of seed embryogenesis and might participate in the highest ACO activity and ethylene production by seeds at the beginning of embryogenesis. Similarly, ethylene is known to regulate somatic embryogenesis of some species. It was found to affect negatively the *Picea glauca* (Moench.) Voss somatic embryo development (Kong and Yeung 1994). El Meskaoui and Tremblay (2001) proved that negative or positive effect of endogenous ethylene on somatic embryo production depended on the capacity of embryogenic cell lines of *Picea mariana* (Mill.) B.S.P. Endogenous ethylene has been reported to promote somatic embryogenesis in *Daucus carota* L. (Nissen 1994) and *Coffea canephora* (Hatanaka et al. 1995). It has been suggested that ethylene modulates cotyledon expansion in microspore-derived embryos of *Brassica napus* (Hays et al. 2000).

There are few studies focused only on the role of ethylene in SE of *Medicago sativa* L. and other *Medicago* species. Early research on the possible involvement of ethylene in the regulation of SE in *Medicago sativa* L. cv. falcata conducted in a two-phase (induction and differentiation) system showed that inhibitors of ethylene biosynthesis (aminooxyacetic acid–AOA, aminoethoxyvinylglycine–AVG, 2,4-dinitrophenol–DNP, salicylic acid–SA) inhibit somatic embryo formation, although contrary to numerous reports, these inhibitors did not affect the rate of ethylene biosynthesis (Meijer and Brown 1988). Further experiments with cobalt and nickel ions, AOA and AVG, all potent inhibitors of ethylene biosynthesis, demonstrated that the high rates of ethylene production during embryo induction are not to be essential for subsequent embryo differentiation (Meijer 1989; Kępczyński et al. 1992). Additionally, Kępczyński et al. (1992) demonstrated that application of 2,5-norbornadiene (NBD), a specific binding inhibitor, to block ethylene action, negatively affected the induction and growth of embryos and their somatic production, but not SE induction in the two-phase system used. When using a regeneration system in which two main phases of SE, induction and differentiation, are separated by an additional step of embryogenic tissue proliferation, we observed that ethylene production during callus growth, proliferation of cell suspension and embryo development in *Medicago sativa* L. cv. Rangelander are greatly altered during SE (Kępczyńska et al. 2009a). Ethylene accumulation in vessels was correlated with the ability of tissues to produce ACC and to convert it into ethylene. It was also established that, apart from induction, each SE phase, particularly embryo development, required a strictly defined level of endogenous ethylene. In their research on *Medicago truncatula* Gaertn., Mantiri et al. (2008) also observed that increasing the level of ACC, an ethylene biosynthesis precursor, by its exogenous application or inhibition of polyamine biosynthesis, which consumed ACC, caused a marked increase of somatic embryo production. Besides, they showed that ACC synthase and ACC oxidase genes were expressed at consistently higher levels in embryogenic tissue of the somatic embryos compared with non-embryogenic callus. An effect of methylglyoxal bis(guanylhydrazone) (MGBG), a polyamine synthesis inhibitor, due to its promotion of ethylene production via increase in the ACC level and ACC synthase activity during an early phase of SE on *M. sativa* L. cv. Jin nan, was also reported by Huang et al. (1998). However, our understanding of ethylene function during individual phases of SE is far from satisfactory. Moreover, there are no data which could answer the question whether ethylene biosynthesis and action during SE is required for the vigor maintenance of the obtained embryos.

In the present study, our main objective was to determine whether controlled disturbance of ethylene biosynthesis and perception during SE in *Medicago sativa* L. cv. Rangelander could influence vigor of the embryos in terms

of their germination and conversion (regeneration). Therefore, during proliferation of embryogenic cells and at their differentiation phases, we used (1) non-specific ethylene biosynthesis inhibitors of ACO and ACS: salicylic acid (SA) and aminoethoxyvinylglycine (AVG), respectively, and (2) specific inhibitors of ethylene binding: 1-methylcyclopropene (1-MCP), 2,5-norbornadiene (NBD). We also determined how the modification of ethylene biosynthesis and perception could affect embryogenic cell growth as well as embryo production and development, and in effect, their ability to germinate and convert.

## Materials and methods

### Plant material

Mother plants of *Medicago sativa* L. cv. Rangelander were grown in soil in a plant growth chamber at 24 ± 2°C, 80% humidity under a 16-h photoperiod with approximately 120 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD). Petioles from fully expanded leaves (second to fourth node from the top of 2- to 3-week-old plants) were excised and served as a source of initial explants. They were surface-disinfected by sequential immersion in 75% ethanol (30 s) and 5% sodium hypochlorite solution (4 min), followed by three times wash in sterile water. Initial explants were prepared as 1-cm-long pieces.

### Somatic embryogenesis

#### *Phase I: callus induction*

For callus induction, the initial petiole explants were cultured for 14 days in 340 mL jars on 40 mL medium SH (Schenk and Hildebrandt 1972) with some modifications (McKersie et al. 1989), containing 4.5 µM 2,4-D; 0.9 µM kinetin; 4.35 mg $L^{-1}$ $K_2SO_4$; 288 mg $L^{-1}$ proline; 53 mg $L^{-1}$ thioproline; 200 mg $L^{-1}$ myoinositol, and solidified with 9 g $L^{-1}$ Lab™ Agar (BioCorp). The cultures were incubated at 25 ± 1°C under a 16-h photoperiod (50 µmol $m^{-2}s^{-1}$ PPFD).

#### *Phase II: embryogenic tissue proliferation*

Suspension cultures were initiated from proliferated calluses (about 1 g fresh weight) by sub-culturing in 100 mL Erlenmeyer flasks on 40 mL liquid $B_5$g, a standard $B_5$ medium (Gamborg et al. 1968) modified to contain 4.5 µM 2,4-D and 0.5 µM NAA for 7 days, at 25°C, 50 µmol $m^{-2}s^{-1}$ PPFD and under a 16-h photoperiod in a refrigerated incubator shaker at 120 rpm (Innova 4340, New Brunswick Sci.). Small embryogenic cell aggregates were isolated by sieving the cell suspension through 500 and 200 µm nylon meshes (Nitex, Sefar AG, Switzerland).

#### *Phase III: differentiation*

For embryo development, the 200 to 500-µm fraction (0.5 mL) was spread on a 200-µm nylon screen placed on hormone-free BOi2Y solid medium (Bingham et al. 1975) containing 0.15 M sucrose (10 mL medium in 60 mm Petri dishes). Two weeks later (38 days after induction), the stages and number of somatic embryos were determined.

#### *Phase IV: maturation of embryos*

After the differentiation, the embryos were transferred together with the nylon screen onto the BOi2Y medium containing 20 µM ABA. When the somatic embryos turned yellow, they were air-dried under sterile conditions and stored in the presence of $K_2CO_3$.

### SA, AVG, 1-MCP and NBD treatments

To perform the treatment, SA and AVG were added separately or together after filtered sterilization at the inception of callus subculture in the $B_5$g medium. SA and AVG solutions were used at pH 5.8 and at concentrations of 1, 10, 100 and 500 µM. To block ethylene receptors, 1-MCP, liberated from powdered SmartFresh™ (Rohm and Haas Co., Italy) was used. The open 4-mL glass vials with 0.14% SmartFresh™ were suspended under the cups of 100 mL Erlenmeyer flasks (proliferation) or 350 mL jars (differentiation phase) equipped with a Parafilm-wrapped rubber stopper. The vials were suspended with threads fixed on screw and flask or jar necks thus were impended about 3 cm below rubber stopper. To these vials the portions of powdered SmartFresh™ were added in amount 0.16, 0.32 and 0.4 mg on 100 mL Erlenmeyer flasks and 5.6, 11.2 and 14 mg on 350 mL jars to obtain after adding of 2 mL water appropriate concentrations $4.5 \times 10^{-8}$, $9.0 \times 10^{-8}$, $1.12 \times 10^{-7}$ M of gaseous 1-MCP. For the NBD experiment, liquid NBD was applied with a Hamilton syringe (7.9, 15.8, 31.5 µL/350 mL jar) through the stoppers onto pieces of filter paper placed under the jar cover. NBD was allowed to evaporate completely and final gas concentrations calculated using Avogadro's law were $2.25 \times 10^{-4}$, $4.5 \times 10^{-4}$, and $0.9 \times 10^{-3}$ M, respectively.

### Ethylene assays

For ethylene assays with suspension cultures in the presence of SA and AVG, the Erlenmeyer flasks were closed with rubber stoppers 24 h prior to the ethylene measurement. Three 1-mL gas samples from the headspace were

collected with a syringe and injected into a gas chromatograph (Hewlett Packard 5890 II) equipped with flame ionization detector and stainless steel column packed with 60–80 mesh Porapak Q. The oven temperature was 60°C. Experiments addressing 1-MCP effects during proliferation or those of 1-MCP and NBD during the differentiation phase, accumulation of ethylene in 100 mL Erlenmeyer flasks or 350 mL jars during 7 or 14 days, respectively, was determined. Ethylene production was expressed as nM/cm$^3$ tissue 24 h$^{-1}$ or nM/cm$^3$ tissue/7 days or/ 14 days.

Germination and conversion of embryos (regeneration)

After 1 week of storage at room temperature, embryos at the cotyledonary stage, picked out at random (20 embryos per replicate), were germinated in the MS medium without hormones (Murashige and Skoog 1962). After 7 days, somatic embryos with visible radicles were scored as germinated, and the conversion rate (in percent) was determined after 2 weeks. The criterion of plant conversion was the presence of roots and of at least one trifoliate leaf.

*Statistical treatment*

Each experiment was run twice in five replicates per treatment. Statistical treatment of the results involved performing the analysis of variance (ANOVA) using the Statistica for Windows version 6.0 software (Statsoft Inc., Tulsa, Oklahoma). Differences between means were considered to be significant at $p = 0.05$, as indicated by Duncan's multiple range test.

## Results

Effects of SA, AVG and SA + AVG applied during the proliferation phase on ethylene production and embryogenic suspension growth

Salicylic acid (SA), an inhibitor of ACC oxidase (ACO) activity, added to the proliferation $B_5$g liquid medium at the lowest concentration (1 μM), had no effect on ethylene production by a 7-day-old embryogenic cell suspension (Fig. 1a). However, SA negatively affected the process at 10, 100 and 500 μM and about 40, 70 and 80% reduction in

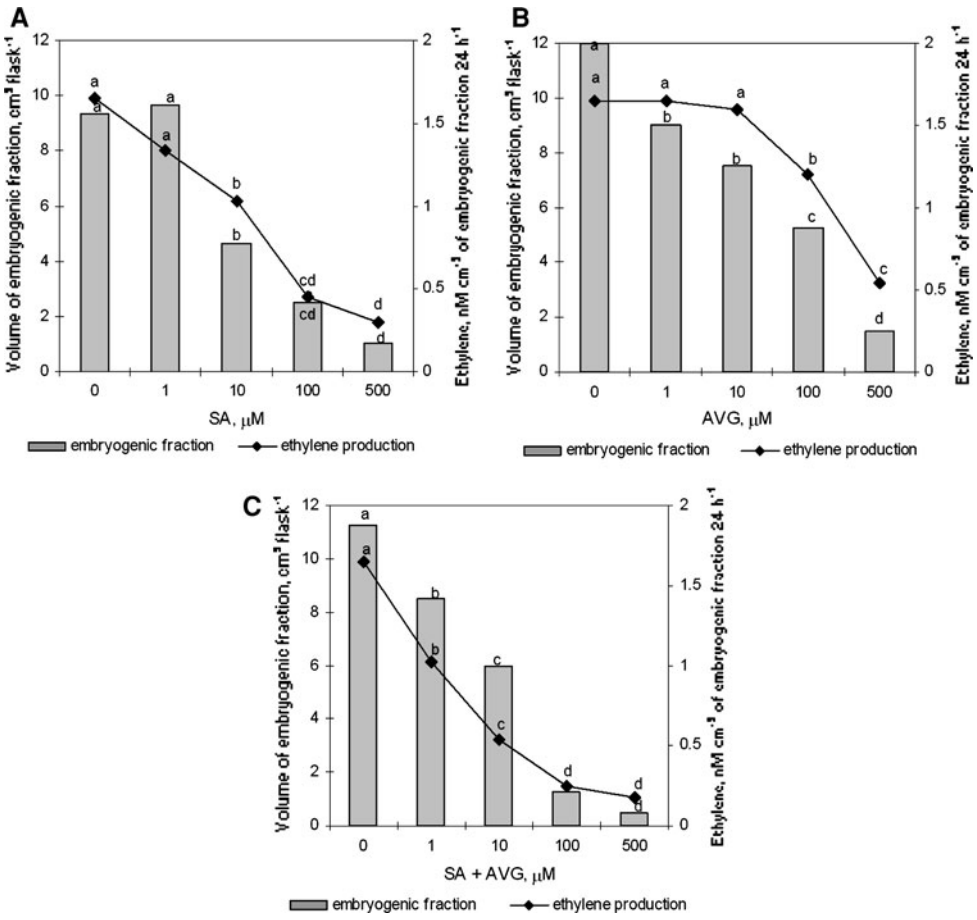


**Fig. 1** Effects of SA (**a**), AVG (**b**) and SA + AVG (**c**) in concentrations of 1, 10, 100, 500 μM added to liquid medium $B_5$g on embryogenic tissue proliferation and ethylene production. Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

ethylene production, respectively, was observed. Inhibitory effect of SA was also observed when the parallel growth of embryogenic cell suspension was considered. In the presence of 1 μM SA, 1 g of callus in the liquid medium produced, during 7 days, an amount of embryogenic fraction identical to that in the control. Application of SA at 10, 100 and 500 μM concentrations resulted in about 50, 70 and 90% inhibition of cell suspension growth, respectively.

Addition of aminoethoxyvinylglycine (AVG), another ethylene biosynthesis inhibitor depressing ACC synthase (ACS) activity, during the same phase at 1 and 10 μM concentrations had no effect on ethylene production (Fig. 1b). On the other hand, AVG concentrations of 100 and 500 μM resulted in about 30 and 70% inhibition, respectively, compared with the control. AVG applied at 1, 10, 100 and 500 μM had a significant inhibitory effect on the cell suspension multiplication rate, reducing it by about 25, 40, 55 and 90%, respectively. In the presence of AVG applied at the highest concentration (500 μM), the cells became albinotic and the suspension was cloudy. Inhibition of ethylene production and embryogenic tissue proliferation due to SA was stronger than that caused by AVG (Fig. 1a, b).

Simultaneous application of SA and AVG to the proliferation medium resulted in a reduction of ethylene production in the presence of all the used concentrations (Fig. 1c). A gradual decline in the ability of the embryogenic cell suspension to produce ethylene was observed. The concentrations of 1, 10, 100 and 500 μM resulted in about 40, 70, 80 and 90% reduction, respectively. The two inhibitors applied at all concentrations negatively affected cell suspension growth; the volume of embryogenic fraction was reduced by about 25, 50, 90 and 95%, respectively.

### Effects of SA, AVG and SA + AVG applied during the proliferation phase on the somatic embryo production and development

Subsequent experiments were aimed at finding out if SA and AVG, applied alone or in combination during the proliferation phase, would affect the embryo production during the next differentiation phase (Fig. 2). The presence of SA at concentrations of 1–500 μM during the proliferation phase had an inhibitory effect on the further embryogenic potential of the suspension culture in terms of the number of somatic embryos produced per Petri dish out of the 0.5 mL embryogenic fraction kept in the hormone-free BOi2Y medium (Fig. 2a). The following inhibition rates were observed: at 1 and 10 μM the inhibition rate was about 20%; 40% inhibition was at 100 μM, and about 70% inhibition was noticed at 500 μM. If the total somatic embryos production is considered, a gradual decline with increasing SA concentrations applied during the proliferation phase was recorded as well: inhibitions by about 25, 70, 80 and below 90% were observed (Fig. 2b). In contrast to SA, AVG added to the proliferation medium significantly inhibited embryo production from the embryogenic fraction in the differentiation medium only at concentrations of 100 and 500 μM, with 10 and 50% reductions being obtained, respectively (Fig. 2c). The total somatic embryo production was inhibited by about 30, 50 and 90% when AVG was applied at 10, 100 and 500 μM, respectively (Fig. 2d). AVG applied simultaneously with SA at all concentrations enhanced the inhibitory effect of SA on the embryogenic potential of the 0.5 mL embryogenic suspension culture to produce somatic embryos (Fig. 2e). A synergic effect of the two inhibitors was observed at concentrations of 100 and 500 μM. The highest inhibitory effect on the total SE production was observed when the two inhibitors were present during the proliferation phase (Fig. 2f).

Somatic embryo development in the differentiation medium was significantly retarded when SA or AVG was added alone or in combination at the earlier phase (Fig. 3). SA at concentrations of 1 and 10 μM significantly (by about 25 and 70%) decreased the number of cotyledonary embryos. At higher concentrations, the compound drastically reduced the development of embryos; its presence at concentrations of 100 and 500 μM in the proliferation medium arrested the embryos during their differentiation phase. Only few embryos at the torpedo stage were observed. Most of the embryos were at the globular stage and the number of globular embryos was 3.6 and 2.6 times higher, respectively, compared with the control (Fig. 3a). AVG, another ethylene biosynthesis inhibitor added to the liquid cell suspension was observed to retard embryo development on the differentiation medium. The effect was significant, although less prominent than that of SA (Fig. 3b).

A twofold increase in the number of globular embryos in the presence of AVG at all concentrations during the proliferation phase occurred. Simultaneously, a significant decrease in the number of cotyledonary embryos, following the treatment involving 1, 10 and 100 μM concentrations was observed, with the reductions amounting to about 25, 35 and 40%, respectively. AVG applied to the cell suspension at the highest concentration (500 μM) retarded the development of the torpedo, cotyledonary and abnormal embryos in the differentiation medium; only globular embryos were present. By comparison, the effect of AVG + SA was most prominent at 1 μM; it was stronger than that obtained when SA and AVG were used alone (Fig. 3c). At higher concentrations of the two inhibitors, the effect was similar to that of applied SA alone.

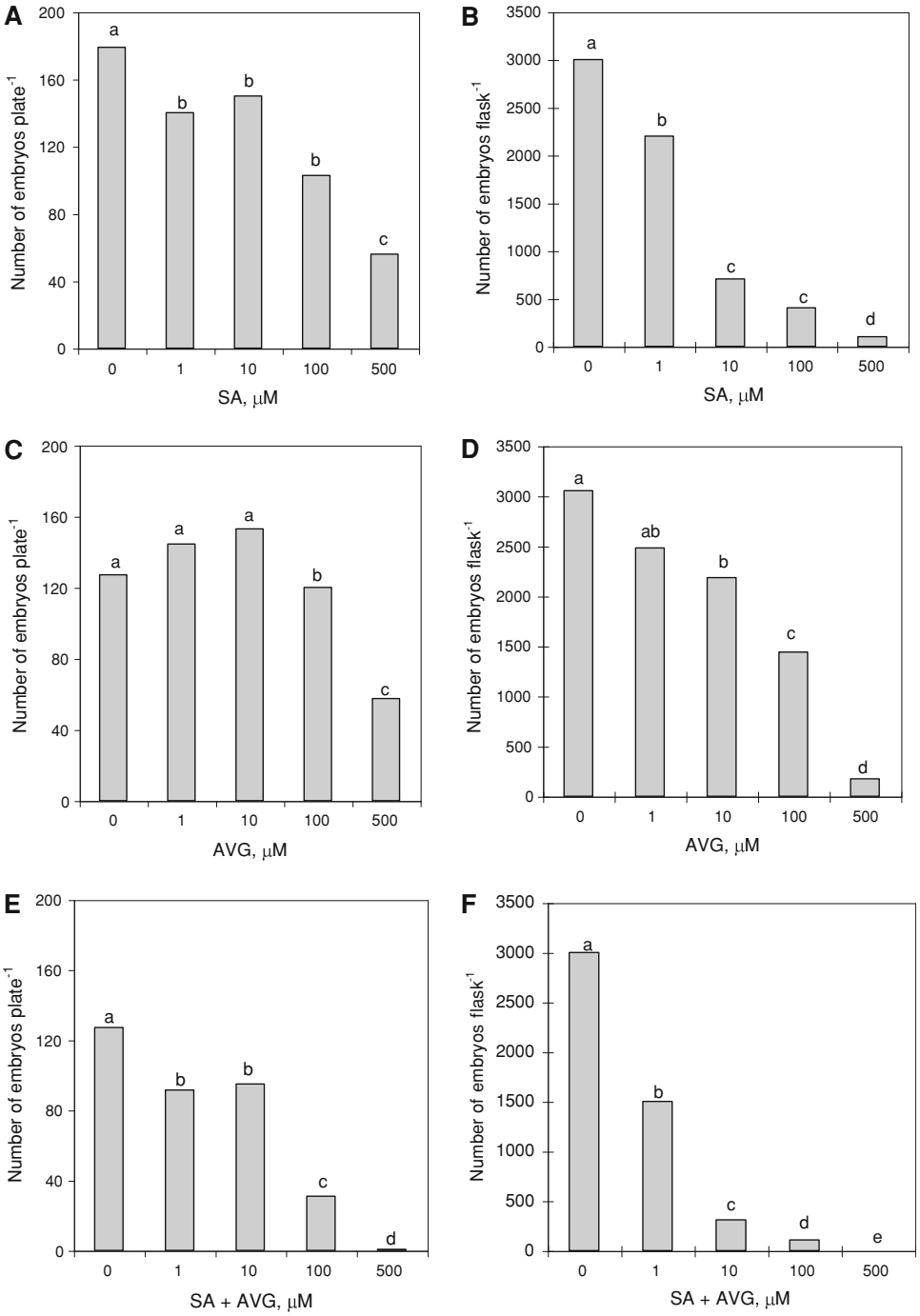


**Fig. 2** Effects of SA (**a**, **b**), AVG (**c**, **d**) and SA + AVG (**e**, **f**) in concentrations of 1, 10, 100, 500 μM added to liquid medium $B_5g$ on embryo production from 0.5 $cm^3$ embryogenic fraction on BOi2Y medium during differentiation phase (**a**, **c**, **e**) and total embryo production from proliferation tissue (**b**, **d**, **f**). Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

Effects of SA, AVG and SA + AVG applied during the proliferation phase on germination and conversion of somatic embryos

All the cotyledonary embryos obtained in the differentiation medium in the experiment described above, after maturation on the ABA-supplemented medium (phase IV of SE) were dried and their ability to germinate and convert on the hormone-free MS medium was tested. The somatic embryos obtained from the embryogenic suspension proliferated in the presence of SA, AVG and SA + AVG demonstrated a significantly reduced ability to germinate (Fig. 4a) and convert (Fig. 4b). Even at the lowest concentration (1 μM) of SA, AVG and SA + AVG, about 50, 30 and 40% reduction in the germination rate was observed, respectively (Fig. 4a). The germination rate was

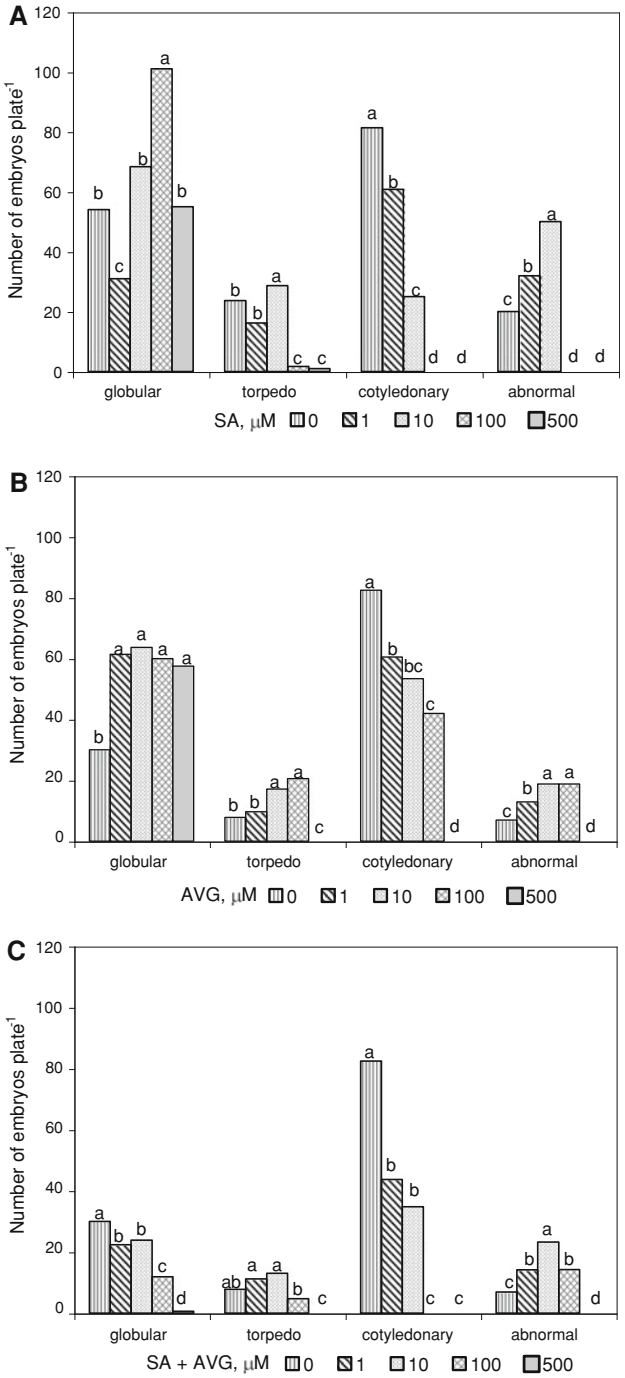


**Fig. 3** Effects of SA (**a**), AVG (**b**) and SA + AVG (**c**) in concentrations of 1, 10, 100, 500 µM added to liquid medium $B_5g$ on further *Medicago sativa* L. somatic embryo development during differentiation phase. Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

observed to decline gradually with increasing concentrations of the inhibitors applied during the proliferation phase, the germination being suppressed at the highest concentration (500 µM). Compared with germination, conversion was more inhibited as a result of the presence of the ethylene biosynthesis inhibitors during the proliferation phase (Fig. 4b). When applied at the lowest concentration (1 µM), SA, AVG and SA + AVG inhibited the further conversion rate by about 70, 50 and 50%, respectively. There was a progressing decline in the conversion of somatic embryos with increasing concentrations of the inhibitors during the proliferation phase. At 500 µM, no somatic embryos could be regenerated into plantlets. Lack of interaction between SA and AVG in inhibition of the regeneration process was observed.

## Effects of the presence of 1-MCP during the proliferation phase on ethylene production, embryogenic suspension growth and subsequent embryo production and development on the differentiation medium

1-methylcyclopropene (1-MCP) was used to determine if ethylene binding to receptors is needed for embryogenic suspension growth and, in consequence, for the further embryo production and development. Presence of 1-MCP in the flasks during proliferation of embryogenic cells resulted in inhibition of ethylene production by the proliferating embryogenic suspension (Fig. 5a). The inhibitor, applied at concentrations of$4.5 \times 10^{-8}$, $9.0 \times 10^{-8}$ and $1.12 \times 10^{-7}$ M (1, 2, 2.5 µL/L, respectively), decreased the rate of ethylene production by the embryogenic fraction by between 50 and 70%, compared with the control. A gradual decline in suspension growth occurred with increasing 1-MCP concentration in the flasks during the proliferation phase and the embryogenic cell suspension growth was reduced by 30, 40 and 60%. Effects of 1-MCP applied during the proliferation phase on production and development of somatic embryos in the differentiation medium are presented in Fig. 5b, c. A gradual decline in the embryogenic potential of the suspension culture, expressed as the number of embryos produced per Petri dish out of the 0.5 mL embryogenic fraction (Fig. 5b), and the total embryo production obtained from the entire embryogenic fraction (Fig. 5c) occurred with increasing concentration of 1-MCP in the flask atmosphere from $4.5 \times 10^{-8}$ to $1.12 \times 10^{-7}$ M. Not only did the presence of the inhibitor during the proliferation phase decrease the number of somatic embryos, but it also inhibited their development (Fig. 5d). 1-MCP at all the tested concentrations was found to slow down the somatic embryo development, resulting in the production of more globular than cotyledonary embryos, when compared wiht the control. It was not possible to test the ability of cotyledonary embryos to germinate in the MS regeneration medium because only few embryos (less than 10) were obtained (Fig. 5d).

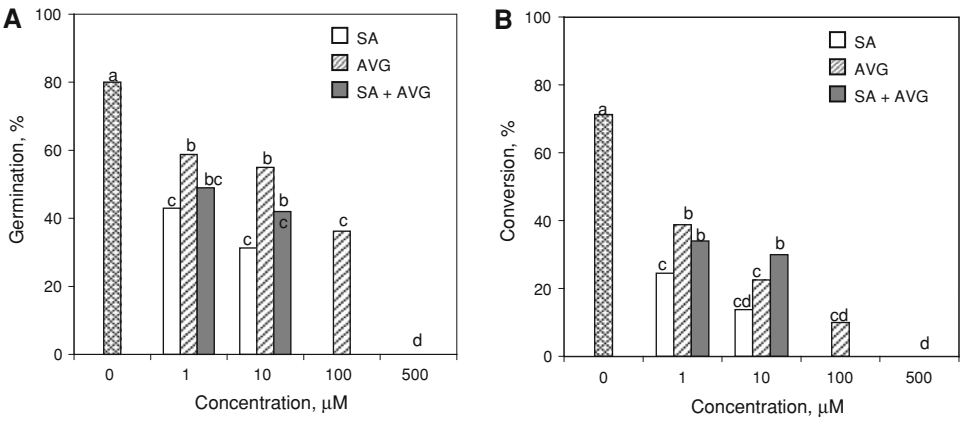


**Fig. 4** Consequence of ethylene biosynthesis inhibitors adding in proliferation phase of indirect somatic embryogenesis in *M. sativa* on further germination (**a**) and conversion (**b**) of mature dehydrated cotyledonary embryos during regeneration phase. Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

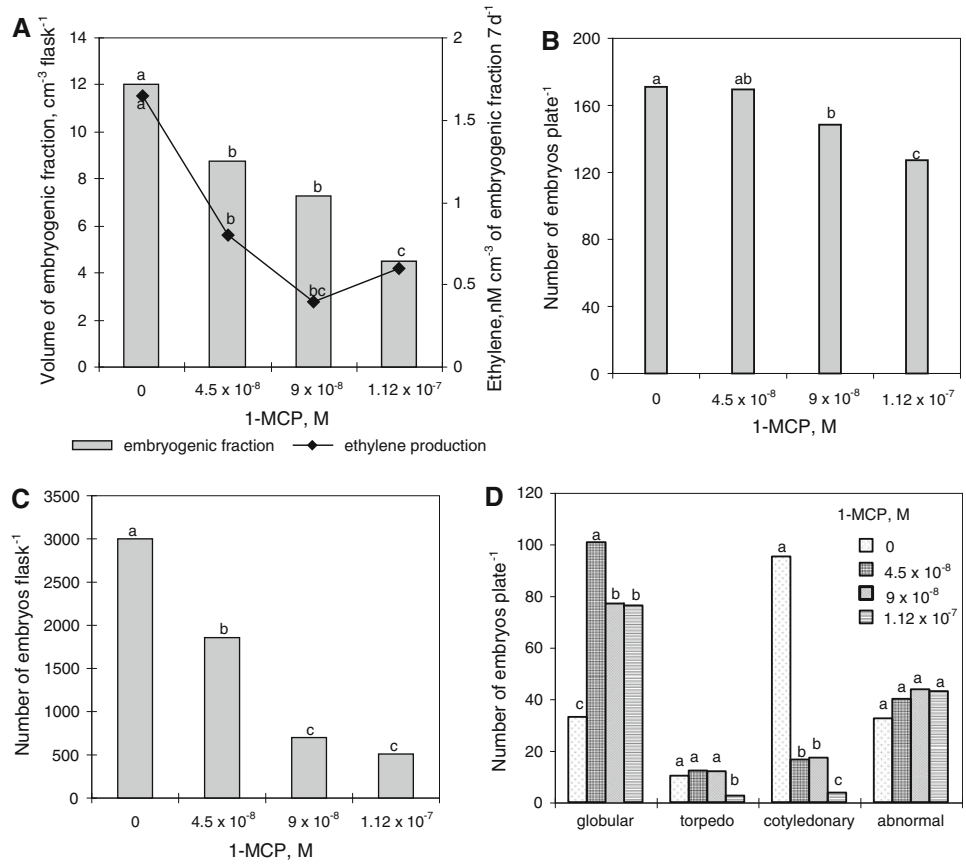


**Fig. 5** The effect of 1-MCP added to liquid medium $B_5g$ in concentrations of $4.5 \times 10^{-8}$, $9 \times 10^{-8}$, $1.12 \times 10^{-7}$ M on embryogenic tissue proliferation and ethylene production (**a**), embryo production from 0.5 $cm^3$ embryogenic fraction during differentiation phase (**b**), total embryo production from proliferation tissue (**c**) and on somatic embryo development in differentiation phase (**d**) of indirect somatic embryogenesis in *M. sativa*. Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

## Effects of the presence of 1-MCP and NBD during the differentiation phase on ethylene accumulation, embryo production and embryo development and regeneration

Because no sufficient number of cotyledonary embryos could be obtained in the experiments involving application of 1-MCP during suspension growth in the liquid medium, the inhibitor was applied at the differentiation phase. It was also of interest to compare its effects with those of NBD, another ethylene action inhibitor (Fig. 6). Usage of 1-MCP at all concentrations reduced ethylene accumulation. The lowest accumulation was observed at 1-MCP concentrations of $4.5 \times 10^{-8}$ and $9 \times 10^{-8}$ M (Fig. 6a). NBD at all used concentrations had no influence on ethylene accumulation. It was observed that the ethylene level was identical to that in the control glass chamber after 14 days. The two ethylene action inhibitors, when applied at all tested concentrations, did inhibit the embryo production (Fig. 6b). A twofold reduction of the process was observed at the highest concentration. Presence of the two inhibitors in ambient atmosphere during the differentiation phase drastically inhibited the embryo development (Fig. 6c). Differentiation of cotyledonary embryos was reduced by

nearly 80% in the presence of all 1-MCP concentrations and at the highest NBD concentration. NBD applied at a lower concentration also caused a high (80%) inhibition of the cotyledonary embryo production. The two ethylene perception inhibitors increased the production of abnormal embryos. The question was addressed whether blocking of ethylene receptors during the differentiation phase would affect the subsequent embryo germination and conversion. Mature dehydrated cotyledonary embryos, developing previously in the presence of 1-MCP and NBD in the differentiation medium, showed a reduced ability to germinate and convert in the regeneration medium (Fig. 6d). Following the treatment with $4.5 \times 10^{-8}$, $9.0 \times 10^{-8}$ and $1.12 \times 10^{-7}$ M 1-MCP during the differentiation phase, somatic embryos germination was reduced by 50–60% and conversion declined by about 60–70%. NBD, another inhibitor of ethylene perception, present at that phase at concentrations of $2.25 \times 10^{-4}$ M, $4.5 \times 10^{-4}$ M and $0.9 \times 10^{-3}$ M, reduced the subsequent germination by about 40–50%, conversion being reduced by 40–60%.

## Discussion

Inhibition of ethylene production by *Medicago sativa* L cv. Rangelander embryogenic cell suspension due to the presence of two non-specific ethylene biosynthesis inhibitors, SA and AVG, during the proliferation phase was correlated with inhibition of cell suspension growth. This may suggest that ethylene is required for cell proliferation. As demonstrated earlier, ethylene production in *Medicago sativa* L. was detected at all SE stages with the production rates depending on the phase and type of regeneration protocol followed: two phases in SE of *M. sativa* cv. falcata (Meijer 1989; Kępczyński et al. 1992), two or four phases in SE of *M. sativa* L. cv. Rangelander (Kępczyńska et al. 2009a). Additionally, we observed that ethylene biosynthesis during transition of embryos between distinct stages of embryo development was more dependent on the availability of ACC than ACO activity (Kępczyńska et al. 2009a). This was in agreement with the results reported by Mantiri et al. (2008) for *Medicago truncatula* Gaertn., where addition of ACC or MGBG, a polyamine biosynthesis inhibitor promoting ACC accumulation, caused a marked increase in the somatic embryo production. Moreover, using AVG, an ACS inhibitor, at 5 and 50 μM concentrations, during proliferation and differentiation, resulted not only in a decline in the total embryo production but also blocked the embryogenic developmental processes. This resulted in the production of more torpedo and globular than cotyledonary embryos, which lends a strong support to the hypothesis of endogenous ethylene involvement in the control of *Medicago sativa* L. cv. Rangelander somatic embryo formation (Kępczyńska et al. 2009a). On the other hand, it is known that ethylene biosynthetic inhibitors may also regulate other biosynthetic pathways which are crucial to somatic embryo development, e.g. polyamines, as suggested by Huang et al. (2001) and Mantiri et al. (2008). Huang et al. (2001) reported that aminooxyacetic acid (AOA), an ACS inhibitor, caused an increase in spermidine and in the number of somatic embryos of *M. sativa* L. cv. Jin nan, but had no effect on ethylene biosynthesis. In the present study, application of both inhibitors alone during the proliferation phase not only brought about a decline in ethylene production and embryogenic suspension proliferation, but also reduced embryo production and inhibited embryonic development, which resulted in the production of more torpedo and globular than cotydelonary embryos. SA was a more effective inhibitor than AVG in all the processes mentioned above. These results may indicate that endogenous ethylene, dependent on ACO and ACS activities, is required during proliferation of embryogenic suspension for the subsequent production of embryos and embryo development in *Medicago sativa* L. cv. Rangelander. Moreover, the above data show that disturbance in ethylene biosynthesis by SA and AVG during cell suspension growth resulted in a loss of ability of obtained cotyledonary embryos on differentiation medium and matured on maturation medium to further germination and conversion on regeneration medium. The inhibitory effect of SA on embryo regeneration was again stronger than that of AVG, although the lack of interaction between SA and AVG in the inhibitory effect was observed. The more pronounced inhibitory effect of SA, compared with that of AVG, can probably be associated with low specificity of that compound. SA, a natural plant growth regulator, not only inhibits ethylene biosynthesis by blocking ACO activity (Leslie and Romani 1988), but also inhibits biosynthesis of jasmonates by depressing allene oxide synthase (AOS) (Harms et al. 1998). As revealed by our previous studies, endogenous JA levels detected in embryogenic suspension (low level) and in the developing embryos (high level) are essential for proembryogenic proliferation and somatic embryo formation, respectively (Ruduś et al. 2009). Although exogenous methyl jasmonate did not influence ethylene production and ACO oxidase activity by the embryogenic suspension, its application during the proliferation phase negatively affected growth of embryogenic suspension as well as subsequent production and development of somatic embryos of *M. sativa* L. (Ruduś et al. 2006; Kępczyńska et al. 2009b). Moreover, as demonstrated by experiments involving indoprofen, an inhibitor of jasmonates, some distinct endogenous level of jasmonates is essential for somatic embryo formation (Ruduś et al. 2006). Thus, summing up these results, it may be

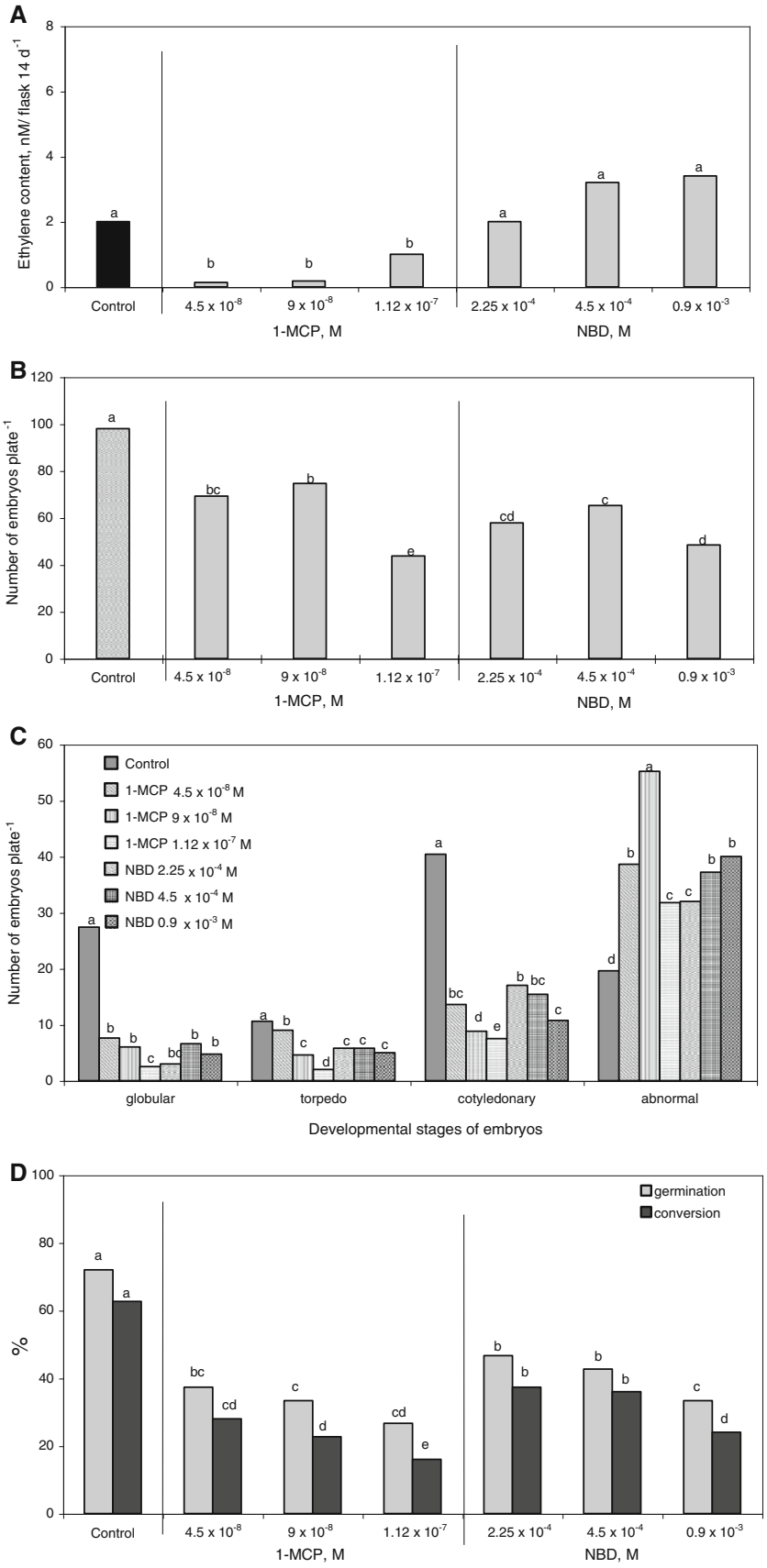


**Fig. 6** Effects of presence of ethylene action inhibitors (1-MCP, NBD) during 14 days' differentiation phase of indirect somatic embryogenesis in *M. sativa* on ethylene content (**a**), embryo production (**b**), embryo development (**c**) and further germination and conversion of mature dehydrated cotyledonary embryos during regeneration phase (**d**). Means with common letters are not significantly different at $p = 0.05$, according to Duncan test

suggested that synthesis of ethylene and jasmonates are required to control the above-mentioned processes in *Medicago sativa* L. cv. Rangelander. However, this line of evidence should be followed at the molecular level. Early studies on the involvement of ethylene biosynthesis in SE regulation in *M. sativa* L. cv. falcata, conducted in a regeneration system with two distinct phases: induction and differentiation, showed non-specific inhibitors of ethylene biosynthesis to be potent inhibitors of somatic embryo formation (Meijer and Brown 1988). Nevertheless, contrary to numerous reports, they (AVG at 25 μM, SA at 20 μM) did not affect the rate of ethylene biosynthesis or increased ethylene production (SA at 10 μM). SA at concentrations of 1–100 μM has been reported to inhibit ethylene production in pear (*Pyrus communis*) cell suspension cultures by blocking the conversion of ACC to ethylene (Leslie and Romani 1986). In carrot somatic embryogenesis, AVG at 0.1 and 1 μM inhibited ethylene and embryo production (Nissen 1994). However, SA inhibited ethylene production, but stimulated somatic embryogenesis in the carrot line used by Roustan et al. (1990). Completely different results were reported by Nissen (1994): SA at concentrations of 1 to 500 μM stimulated ethylene accumulation and inhibited embryo production.

To find out whether ethylene action is required during SE and for vigor maintenance of the somatic embryos obtained, we blocked ethylene receptors with 1-MCP during the proliferation or used both 1-MCP and NBD during the differentiation phases. As a result of the 1-MCP treatment during the proliferation phase, not only was the ability of embryogenic cell suspension to produce ethylene reduced and the embryonic fraction growth decreased, but also, as a consequence, the embryogenic cells showed a reduced ability to produce somatic embryos in the differentiation medium. The effect of blocking ethylene receptors by 1-MCP was particularly distinct in the development of embryos, whereby only few cotyledonary embryos were obtained. It must have been the reason why the vigor, in terms of germination and conversion, of the embryos obtained could not be determined.

During the differentiation phase, the absence of available ethylene to the receptors, caused by 1-MCP treatment adversely affected the ethylene and embryo production and impacted embryo development as well. The treatment resulted in a strong reduction of somatic embryos vigor; particularly affected was the conversion to seedlings, reduced by about 55% to as much as 75%, depending on the concentrations used. To verify that blocking of ethylene binding can destroy somatic embryo production, development and the ability to regenerate, NBD, another ethylene binding inhibitor, was used during differentiation. NBD had no effect on ethylene production, but reduced the embryo production and development. This is in agreement with earlier findings of Kępczyński et al. (1992) who reported that the inhibition of ethylene binding by NBD during the differentiation stage inhibited *Medicago sativa* embryo production and differentiation. Thus, the experiments with 1-MCP and NBD suggest that binding of ethylene is important for embryo production and development. This contention is in agreement with the results obtained in the experiment with SA and AVG, which demonstrated biosynthesis of ethylene to be required by the aforementioned processes. Moreover, blocking of ethylene binding during differentiation had a negative effect on the subsequent vigor of somatic embryos, expressed as the ability to germinate and convert in the regeneration medium.

In conclusion, the present study provides an additional support to our earlier suggestion of the importance of ethylene biosynthesis during proliferation of embryogenic suspension and embryo development in *Medicago sativa* L. cv. Rangelander. Moreover, blocking of ethylene binding by 1-MCP and NBD disturbed the above-mentioned morphological processes and indicated that not only biosynthesis, but also ethylene binding to receptors was required to keep those processes on the appropriate course. Till date, this is the first report on usage of 1-MCP during different phases of SE and the first information that disturbance in ethylene biosynthesis and binding during proliferation of embryogenic suspension and embryo development in *Medicago sativa* L. cv. Rangelander, negatively impacted germination and conversion of cotyledonary embryos, i.e. their vigor. However, further studies are required for unequivocal explanation of mechanism of ethylene involvement in the establishment of embryo quality in *Medicago sativa*.

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