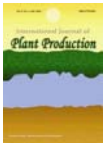

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Effect of plant-derived smoke extract on *in vitro* plantlet regeneration from rapeseed (*Brassica napus* L. cv. Topas) microspore-derived embryos

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Abstract

The effect of aqueous smoke extract, derived from *Tanacetum parthenium*, on *in vitro* plantlet regeneration from rapeseed (*Brassica napus* L. cv. Topas) microspore-derived embryos (MDEs) was evaluated in this study. Inoculation of rapeseed MDEs with the smoke-extract dilutions (1:250, 1:500, 1:1000 and 1:2000) for various times (5, 10 and 15 min) significantly enhanced the percentage of regenerated plantlets from these embryos and also decreased the callogenesis percentage in these explants. The root length and shoot length of inoculated MDEs were significantly improved with all smoke-extract dilutions except for dilutions of 1:100 and 1:10. Application of smoke-extract solutions (1:1000, 1:500, 1:250 and 1:100) in B_5 regeneration medium significantly enhanced the plantlet regeneration from rapeseed MDEs compared to control cultures. Furthermore, use of smoke-extract solutions in combination with GA_3 (0.1 and 0.15 mgl^{-1}) significantly improved the root length and shoot length of rapeseed MDEs compared to separate use of smoke-extract solutions. Use of filter paper saturated with aqueous smoke extract (1:250) on top of the B_5 regeneration medium was the best smoke treatment method improving the plantlet regeneration percentage, root length and shoot length of rapeseed MDEs compared to other treatment methods. The conversion frequency of rapeseed MDEs pretreated with smoke extract dilution of 1:500 was improved up to 50% compared with the control MDEs (26.67%), whereas the use of smoke extract (1:100) in B_5 regeneration medium enhanced the conversion frequency of these MDEs up to 62% compared with the control cultures (26.67%).

Keywords: *Brassica napus* L.; Plant-derived smoke; Plantlet regeneration; Microspore-derived embryo.

Abbreviations

MDE Microspore-derived embryo, GA Gibberellic acid, PGR Plant growth regulator

Introduction

Plant-derived smoke generated by burning is widely recognized as an important germination stimulant. After the discovery by De Lange and Boucher (1990) that seeds of *Audouinia capitata* Brongn. germinate well at sites with smoke-fumigated soils, but not at smoke-free (control) sites, numerous studies have revealed the potential of plant-derived smoke in many aspects of plant biology such as seed germination, seedling growth (Kulkarni et al., 2005; Kulkarni et al., 2007; Kulkarni et al., 2008; Abdollahi et al., 2011), plant flowering (Keeley, 1993), root initiation and development (Taylor and Van Staden, 1996), somatic embryogenesis and embryo development (Senaratna et al., 1999; Ma et al., 2006, Malabadi and Nataraja, 2007). The active germination stimulant in smoke, now known as karrikinolide (Nelson et al., 2009), was identified to be the butenolide, 3-methyl-2H-furo [2, 3-c] pyran-2-one (Flematti et al., 2004). It was suggested that the active compound (s) in smoke could behave in a manner similar to that of other plant growth regulators (PGRs) (Senaratna et al., 1999; Ma et al., 2006) and affect the plant processes through interaction with endogenous PGRs such as gibberellins, cytokinins, abscisic acid and ethylene in photoblastic and thermodormant seeds (Van Staden et al., 2000).

Brassicaceae is a plant family that showed positive response to smoke treatment (Brown et al., 2003), and *Brassica napus* known as rapeseed is a flowering member of this family. In Iran, rapeseed is edible oil crop and has recently been exploited to boost its production (Amiri-Oghan et al., 2009). There is a little or no information about the impact of plant-derived smoke on germination and growth of rapeseed plants *in vitro* and *in vivo*. Thornton et al. (1999) only described the promotive effect of combustion products from plant vegetation on the release of rapeseed seeds from dormancy. In the study of these researchers, the combustion product solution (1:100 dilution) from willow (*Salix viminalis*), *Themeda triandra* leaves and wheat (*Triticum sativum*) broke dormancy of rapeseeds in the dark at 20 ^{o}C and also enhanced the seedling growth of rapeseed in comparison to that of the dark water controls.

Microspore-derived embryos (MDEs) are important materials for plant breeders to generate haploid and doubled haploids plants, which have

considerable value in plant breeding (Evans et al., 1990). Microspore culture and plant regeneration of rapeseed (*B. napus*) has been investigated extensively because of its high frequency of androgenesis (Pechan and Keller, 1988). However, one problem with the practical application of MDEs in *B. napus* breeding is that these androgenic embryos convert to plantlets directly but only at low frequencies (1-47%) (Kott and Beversdorf, 1990). Generally, all plant materials are suitable for preparation of smoke extracts with stimulatory effects in plant growth process (Jäger et al., 1996). As the smoke derived compounds stimulate seed germination, it was questioned whether they are equally effective in enhancing *B. napus* plantlet regeneration from MDEs. Thus, in the present study, the effects of aqueous smoke extracts derived from burning leaf material of *Tanacetum parthenium* (a traditional medicinal herb), alone or in combination with GA_3 was tested for *in vitro* plantlet regeneration and growth properties of rapeseed MDEs.

Material and Methods

Plant material and growth condition

A spring cultivar of rapeseed (*Brassica napus* cv. Topas), competent for microspore embryogenesis was used in this study. Seeds were kindly provided by Dr. J.M. Segui-Simarro (institute of COMAV, Spain). The donor plants for microspore culture were grown under growth cabinet conditions with a 16 h photoperiod, a day/night temperature of 15/10 ^{o}C and a light intensity of 300 μmol m^{-2}.

Microspore culture

The microspore culture was based on protocol of researchers (Haddadi et al., 2008; Abdollahi et al., 2009). About 50-100 flower buds (2.5 to 3.5 mm) at the late uninucleate stage and early binucleate stage of microspore development were selected. The buds were surface sterilized in approximately 100 ml of 5.25% sodium hypochlorite for 10 min with agitation prior to washing three times (5 min for each time) with sterile water. The buds were then macerated in a cold microspore isolation solution (Fletcher et al., 1998), distilled water containing 13% sucrose and buffered at pH 6.0. This microspore suspension was filtered through a 106 μm metal sieve followed by a 53 μm metal sieve. The crude microspore suspension

was centrifuged at 181 g for 5 min, the supernatant was decanted, and 5 ml cold microspore isolation medium was added to resuspend the pellet. This procedure was repeated twice for a total of three washes. Before the last centrifuge spin, the number of microspores was determined using a hemacytometer, and then microspores were resuspended in NLN-13 medium (Lichter, 1982) with 13% sucrose, pH 6.0. A microspore density of about 4×10^4 per ml was used in this study. The microspore suspension was then dispensed into 100×15 mm Petri dishes, 10 ml in each. The dishes were sealed with double layers of Parafilm, incubated at 30±0.5 ^oC in the dark for 14 days and then moved to 25 ^oC still in the dark on a shaker (40 rpm). After 35 days, MDEs at cotyledonary stage were transferred to B_5 regeneration medium (Gamborg et al., 1968), containing 0.1 mgl^{-1} Gibberellic acid (GA_3), 20 mgl^{-1} sucrose and 8 gl^{-1} agar, pH 5.7.

Smoke-extract solutions

In this study, the dry leaf material of *T. parthenium* (5 kg) was burnt in a 20-L metal drum, and smoke generated was passed through a glass column containing 500 ml of tap water for 45 min (Baxter et al., 1994). This smoke water was filtered through Whatman No. 1 filter paper. A neutral fraction of smoke water, without the weaker acids, phenols and stronger acids was prepared by a protocol involving the ether, NaOH soluble and $NaHCO_3$ soluble (Flematti et al., 2007) and used as stock solution. The test solutions were prepared by diluting 1 ml of this neutral smoke-extract with different volumes (v/v) of distilled water or culture media. Smoke-extract solutions were sterilized using an autoclave (121 ^oC and 1.2 bar) for 20 min.

Plantlet regeneration experiments with rapeseed MDEs

Three separate experiments were conducted to examine the effect of plant-derived smoke solutions on in vitro plantlet regeneration and growth properties in rapeseed MDEs. In the first experiment, rapeseed MDEs (35-day-old) were inoculated with smoke-extract dilutions of 1:10, 1:100, 1:250, 1:500, 1:1000, 1:2000 and control distilled water for 5, 10 and 15 min. Inoculated MDEs were rinsed two times (5 min) with sterile distilled water and dried on a sterile filter paper. Rapeseed MDEs were then transferred to B_5 regeneration medium, containing 0.1 mgl^{-1} GA_3, 20 mgl^{-1} sucrose and 8 gl^{-1} agar, pH 5.7. The experimental design was a completely

randomized factorial (7×3) with three replications, each consisting of one Petri dish (100×15 mm) with 10 explants (MDEs). In second experiment, the combined effect of smoke-extract dilutions and GA_3 on the plantlet regeneration and growth of rapeseed MDEs was investigated. In this experiment, smoke-extract dilutions were made using the crude smoke extracts in the B_5 medium and sterilized using an autoclave. This experiment was arranged in a completely randomized design, in a factorial 7×4. Six concentrations of smoke extracts (1:10, 1:100, 1:250, 1:500, 1:1000, 1:2000) and distilled water as control were used with four levels of GA_3 (0, 0.1, 0.15 and 0.2 mgl^{-1}). Each experimental unit (Petri dish) consisted of 10 explants (MDEs) with three replicates. The final test compares the effect of different treatment methods of rapeseed MDEs with smoke extract dilution on plantlet regeneration and growth. Inoculation of rapeseed MDEs with smoke-extract dilution of 1:250 for 15 min, use of smoke extract (1:250) in B_5 regeneration medium, use of filter paper saturated with or without aqueous smoke extract (1:250) on top of the Petri dishes containing the B_5 medium were compared to control (untreated MDEs were transferred to B_5 regeneration medium without any smoke extract). A completely randomized design with three replications was used in this experiment

Data analysis

All data was analyzed using SPSS statistical package (version 16, SPSS Inc., an IBM Company, USA) and means using Duncan's test at $P<0.05$ were compared. Three replicates were used for each treatment, in which 10 MDEs placed in a Petri dish containing the B_5 regeneration medium was considered as a replication. All experiments were repeated thrice and the results were presented based on the average of the three experiments.

Results and Discussion

Interaction between smoke-extract dilutions and inoculation times on plantlet regeneration and growth properties of rapeseed MDEs

The interaction between different smoke-extract dilutions and inoculation times of rapeseed MDEs was not statistically significant ($P<0.05$) for plantlet regeneration and callogenesis percentages, while there was a

significant differences between various smoke-extract dilutions for percentages of plantlet regeneration and callogenesis (Table 1). The smoke-extract dilutions of 1:250, 1:500 and 1:1000 significantly enhanced the percentage of plantlet regeneration compared to control and other smoke-extract treatments. These smoke-extract dilutions showed the highest percentages of plantlet regeneration compared to control and other dilutions of smoke extract (Table 2, Figure 1A).These concentrations of smoke extract significantly reduced the percentage of callogenesis in MDEs compared with the higher concentrations (1:100 and 1:10), but not control cultures (Table 2). The smoke-extract dilutions of 1:10 and 1:100 had a significant negative effect or no significant effect on the percentage of plantlet regeneration compared to control distilled water (Table 2).

Table 1. Analysis of variance summary for plantlet regeneration traits of rapeseed MDEs inoculated with smoke-extract dilutions for various times.

Source of Variation	Df	Mean Square			
		Plant regeneration (%)	Callogenesis (%)	Root length	Shoot length
Smoke-extract concentration (A)	6	1575.661^{***}	106.878^{**}	40.714^{***}	4.937^{***}
Inoculation time (B)	2	68.254^{ns}	0.000^{ns}	3.398^{***}	1.764^{***}
A×B	12	58.995^{ns}	20.370^{ns}	1.346^{***}	0.896^{***}
Error	42	44.444	28.571	0.051	0.058

** P<0.01; *** P<0.001; ^{ns} Non significant.

Table 2. The effect of different smoke-extract dilutions (used at explant) on plantlet regeneration and callogenesis percentage in rapeseed (*Brassica napus* L.cv. Topas) MDEs after 4 weeks of culture in B_5 regeneration medium under 16:8 h light/dark period at 25±0.5 ^{o}C.

Smoke extract dilutions	Mean ± SD (%)	
	Plantlet regeneration	Callogenesis
Control	26.67 ± 5.00^{c}	20.00 ± 0.00^{ab}
1:2000	33.33 ± 8.66^{b}	20.00 ± 5.00^{ab}
1:1000	46.67 ± 7.07^{a}	15.56 ± 5.27^{b}
1:500	50.00 ± 5.00^{a}	16.67 ± 5.00^{b}
1:250	48.89 ± 7.82^{a}	16.67 ± 5.00^{b}
1:100	25.56 ± 5.27^{c}	24.44 ± 5.27^{a}
1:10	16.67 ± 8.66^{d}	23.33 ± 7.07^{a}

Means with the same letter (s) are not significantly different from each other.

Interaction between smoke-extract dilutions and inoculation times of rapeseed MDEs was statistically significant ($P<0.001$) for root length and shoot length (Table 1). Inoculation of rapeseed MDEs with smoke-extract dilution of 1:250 for 15 min was the best treatment for improvement of root length in regenerated plantlets (Table 3, Figure 1B). Concerning the shoot length of regenerated plantlets, inoculation of MDEs with smoke-extract dilution of 1:250 (10 min) and dilutions of 1:500 and 1:250 (15 min) were the best smoke treatments and showed the highest shoot length compared to control and other smoke treatments (Table 3, Figure 1B). Inoculation of rapeseed MDEs with smoke-extract dilution of 1:10 for 15 min showed a negative effect on root length compared to control.

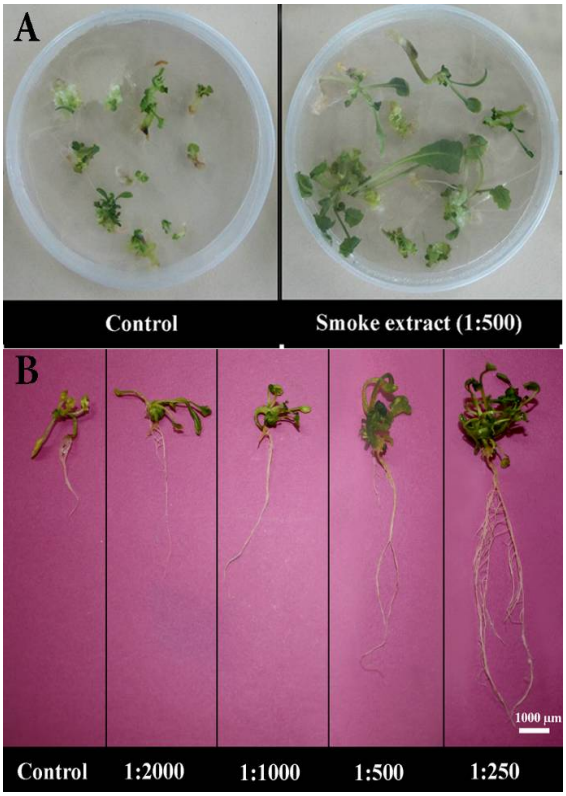

Figure 1. Improvement of plantlet regeneration percentage in rapeseed (*B. napus* L. cv. Topas) MDEs inoculated with smoke-extract dilution of 1:500 (A), and the effect of different smoke-extract dilutions on root length and shoot length of regenerated plantlets from rapeseed (*B. napus* L.cv. Topas) MDEs after 4 weeks of culture in B_5 regeneration medium under 16:8h light/dark period at 25±0.5 ^{o}C (B). The surface dried MDEs of rapeseed were inoculated with smoke-extract dilutions for various periods (5, 10 and 15 min).

Table 3. The interaction between smoke-extract dilutions (used at explant) and inoculation times on root and shoot length of rapeseed (*Brassica napus* L.cv. Topas) MDEs after 4 weeks of culture in B_5 regeneration medium under 16:8h light/dark period at 25±0.5 °C.

Smoke extract dilutions (v/v)	Inoculation times (min)	Mean ± SD	
		Root Length (cm)	Shoot Length (cm)
Control	5	5.13 ± 0.21^{g}	2.07 ± 0.15^{d}
	10	5.00 ± 0.17^{g}	2.03 ± 0.31^{d}
	15	6.10 ± 0.26^{f}	3.07 ± 0.15^{c}
1:2000	5	5.97 ± 0.12^{f}	3.03 ± 0.21^{c}
	10	7.03 ± 0.21^{e}	3.00 ± 0.26^{c}
	15	7.07 ± 0.32^{e}	4.07 ± 0.15^{b}
1:1000	5	7.07 ± 0.15^{e}	4.03 ± 0.21^{b}
	10	7.93 ± 0.25^{d}	3.03 ± 0.21^{c}
	15	8.20 ± 0.17^{d}	4.07 ± 0.15^{b}
1:500	5	7.97 ± 0.32^{d}	4.03 ± 0.31^{b}
	10	8.10 ± 0.26^{d}	4.07 ± 0.35^{b}
	15	8.03 ± 0.21^{d}	5.03 ± 0.31^{a}
1:250	5	9.03 ± 0.21^{c}	4.07 ± 0.25^{b}
	10	11.07 ± 0.25^{b}	5.07 ± 0.15^{a}
	15	12.03 ± 0.31^{a}	4.97 ± 0.38^{a}
1:100	5	5.17 ± 0.12^{g}	3.07 ± 0.25^{c}
	10	4.93 ± 0.15^{g}	4.03 ± 0.21^{b}
	15	5.20 ± 0.10^{g}	4.07 ± 0.25^{b}
1:10	5	4.83 ± 0.32^{g}	4.23 ± 0.06^{b}
	10	5.10 ± 0.20^{g}	3.97 ± 0.32^{b}
	15	3.97 ± 0.21^{h}	3.07 ± 0.15^{c}

Means with the same letter (s) are not significantly ($P<0.001$) different from each other.

The stimulatory effect of plant-derived smoke on seedling growth properties and rooting was previously proved by other researchers (Taylor and Van Staden, 1998; Ma et al., 2006; Abdollahi et al., 2011).

In a previous study, incorporation of butenolide into the culture medium reduced the period of embryo development and improved the frequency of root formation in *Baloskion tetraphyllum* (Ma et al., 2006). Aqueous smoke extracts derived from both *Themeda triandra* and *Passerina vulgaris* had also a significant promotive effect on the growth of roots in cuttings of *Vigna radiata* (Taylor and Van Staden, 1996). The extracts used in the last study, stimulate rooting over a wide range of dilutions,

and there is no significant inhibition of rooting except for very concentrated applications of the crude extract. These findings support the results of our study that high concentrations of smoke extracts (1:10 and 1:100) showed a negative effect on root length of regenerated plantlets from rapeseed MDEs.

Combined effect of smoke-extract solutions (used in culture medium) and GA_3 on plantlet regeneration and growth properties of rapeseed MDEs

In this experiment, the interaction between the smoke-extract solutions used in B_5 regeneration medium and GA_3 was not statistically significant ($P<0.05$) for percentages of plantlet regeneration and callogenesis (Table 4). Different smoke-extract dilutions used in B_5 regeneration medium significantly ($P<0.001$) enhanced the percentage of plantlet regeneration from rapeseed MDEs (Table 5). Use of smoke-extract dilution of 1:100 in culture medium resulted in highest percentage of plantlet regeneration compared to control and other smoke treatments (Table 5). The effect of GA_3 on percentage of plantlet regeneration from rapeseed MDEs was statistically significant ($P<0.01$, Table 4). Use of $0.1 mgl^{-1}$ GA_3 in B_5 regeneration medium showed the highest percentage of plantlet regeneration compared to control and other levels of GA_3 (Table 5). Different concentrations of smoke-extracts and various levels of GA_3 did not show significant effect ($P<0.05$) on percentage of callogenesis in rapeseed MDEs (Table 4).

Table 4. Analysis of variance summary for plantlet regeneration traits of rapeseed MDEs treated with different concentrations of smoke-extract and GA_3 in B_5 regeneration medium.

Source of Variation	Df	Mean Square			
		Plant regeneration (%)	Callogenesis (%)	Root length	Shoot length
GA_3 concentration (A)	3	241.667^{**}	10.556^{ns}	18.530^{***}	10.713^{***}
Smoke-extract concentration (B)	4	2154.167^{***}	106.667^{ns}	27.983^{***}	9.969^{***}
A×B	12	54.167^{ns}	24.444^{ns}	1.533^{***}	0.929^{***}
Error	40	43.333	46.667	0.140	0.124

** $P<0.01$; *** $P<0.001$; ^{ns} Non significant.

Table 5. The effect of different concentrations of smoke-extracts and GA_3 (used in B_5 regeneration medium) on plantlet regeneration (%) from rapeseed (*B. napus* L.cv. Topas) MDEs after 4 weeks medium under 16:8h light/dark period at 25 ± 0.5 °C.

Treatment		Mean ± SD (%)
		Plantlet regeneration
Smoke-extract solution (v/v)	Control	26.67 ± 4.92[d]
	1:1000	45.83 ± 9.00[c]
	1:500	47.50 ± 6.22[c]
	1:250	55.00 ± 10.00[b]
	1:100	62.50 ± 6.22[a]
Gibberellic acid (mgl^{-1})	0	44.00 ± 13.52[b]
	0.10	52.67 ± 16.24[a]
	0.15	48.67 ± 14.07[ab]
	0.20	44.67 ± 11.87[b]

Means with the same letter (s) are not significantly different from each other.

In this experiment, smoke-extract (concentration of 1:100) showed the highest percentage of plantlet regeneration (62.50%) compared with the control (26.67%) and with respect to other concentrations of the smoke-extracts (Table 5), whereas in the first experiment, when the rapeseed MDEs inoculated with smoke-extract dilutions, this concentration (1:100) reduced the percentage of plantlet regeneration (25.56%) compared to the control and other concentrations of smoke-extract except for 1:10 (Table 2). These facts are suggesting that smoke extracts may also include toxic substances with detrimental effects on plant growth process, as also proposed by several authors (Senaratna et al., 1999; Light et al., 2002; Light et al., 2009). This way, when smoke extracts are applied as a pretreatment (inoculated MDEs), the stimulating effects would be evident, but so would be the negative, deleterious effects (concentrations of 1:100 and 1:10). When smoke extracts are added to the culture medium, the rest of compounds would somehow avoid such effects, possibly inactivating or buffering potentially toxic substances. Thus, regeneration medium would allow for the use of higher extract concentrations with no detrimental effects (Table 5).

Interaction effect between the smoke-extracts and GA_3 in B_5 regeneration medium on root and shoot length of regenerated plantlets from rapeseed MDEs, was significant (Table 4). Combined use of smoke extract (1:100) and 0.1 mgl^{-1} GA_3 in B_5 regeneration medium was the best treatment for improvement of root length and shoot length compared to control and other treatments (Table 6).

There are several studies that show that smoke extracts exhibit hormone-like responses and shows interaction with PGRs. For example, there is an increase in putative GA measured using the dwarf rice microdrop assay in smoke-treated lettuce seeds compared to red light-treated seeds (Gardner

et al., 2001). Kepczynski et al. (2006) showed that separate application of smoke solution (1:1000 dilution) or GA_3 had no significant effect on germination of *Avena fatua* caryopses after 24 h of incubation in comparison to the control ($P<0.05$). However, combining the GA_3 (10^{-5} and 10^{-4} M) with the smoke solution significantly increased the germination rate and resulted in germination levels of about 80% after 24 h. In light-sensitive *Lactuca sativa* seeds, Van Staden et al. (1995) observed a similar synergism between GA_3 and smoke. It was concluded that gibberellin synthesis could be considered as a possible component of the mechanism involved in smoke-stimulated germination. Furthermore, it was also shown in *Nicotiana attenuata* that smoke exposure increased the sensitivity of the seeds to exogenous GA_3 treatment (Schwachtje and Baldwin, 2004). However, gibberellins, if applied simultaneously with liquid smoke, achieved their maximum effectiveness at concentrations that were several times lower than those where gibberellins were applied alone (Todorovic et al., 2005). These finding are consistent with the results obtained in our study.

Table 6. The interaction between smoke extracts and GA_3 (used in B_5 regeneration medium) on root length and shoot length of regenerated plantlets from rapeseed (*B. napus* L.cv. Topas) MDEs after 4 weeks of culture under 16:8h light/dark period at 25±0.5 ^oC.

GA_3 (mgl^{-1})	Smoke-extract solutions	Mean ± SD	
		Root Length (cm)	Shoot Length (cm)
0	Control	5.40 ± 0.70^{g}	2.53 ± 0.67^{efg}
	1:1000	6.03 ± 0.21^{fg}	1.93 ± 0.25^{h}
	1:500	6.07 ± 0.32^{fg}	2.13 ± 0.25^{gh}
	1:250	6.07 ± 0.15^{fg}	3.17 ± 0.25^{de}
	1:100	8.07 ± 0.15^{d}	4.03 ± 0.31^{c}
0.10	Control	5.37 ± 0.76^{g}	2.60 ± 0.70^{efg}
	1:1000	8.97 ± 0.32^{c}	5.00 ± 0.26^{b}
	1:500	8.97 ± 0.31^{c}	5.07 ± 0.15^{b}
	1:250	9.00 ± 0.26^{c}	5.03 ± 0.31^{b}
	1:100	12.07 ± 0.32^{a}	6.07 ± 0.15^{a}
0.15	Control	5.70 ± 0.61^{fg}	2.57 ± 0.47^{efg}
	1:1000	7.00 ± 0.26^{e}	3.87 ± 0.21^{c}
	1:500	7.13 ± 0.21^{e}	4.07 ± 0.15^{c}
	1:250	6.97 ± 0.32^{e}	3.97 ± 0.21^{c}
	1:100	9.90 ± 0.26^{b}	5.07 ± 0.15^{b}
0.20	Control	5.53 ± 0.58^{fg}	2.47 ± 0.72^{fgh}
	1:1000	6.13 ± 0.21^{f}	3.20 ± 0.17^{d}
	1:500	6.07 ± 0.32^{fg}	3.00 ± 0.26^{def}
	1:250	7.17 ± 0.06^{e}	3.03 ± 0.21^{def}
	1:100	8.93 ± 0.15^{c}	5.17 ± 0.06^{b}

Means with the same letter (s) are not significantly ($P<0.001$) different from each other.

Effect of different treatment methods of rapeseed MDEs with aqueous smoke extract on plantlet regeneration (%) and growth properties

Different treatment methods of rapeseed MDEs with aqueous smoke extract (1:250) were significantly ($P<0.05$) different for percentage of plantlet regeneration (Table 7) while had no significant effect on callogenesis percentage (Table 7). Use of filter paper saturated with aqueous smoke extract (1:250) on top of the B_5 regeneration medium and inoculation of rapeseed MDEs with smoke extract dilution of 1:250 were the best treatment methods and showed the highest percentages of plantlet regeneration compared to control and other treatment methods (Table 8).

Different treatment methods of rapeseed MDEs with smoke-extract dilution of 1:250 also showed significant ($P<0.001$) differences for root length and shoot length (Table 7). Use of filter paper saturated with aqueous smoke extract (1:250) on top of the B_5 regeneration medium was the most effective method improving the root length and shoot length of rapeseed regenerated plantlets compared to control and other treatment methods (Table 8).

Table 7. Analysis of variance summary for plantlet regeneration traits of rapeseed MDEs treated with smoke-extract dilution of 1:250 through different methods.

Source of Variation	Df	Mean Square			
		Plant regeneration (%)	Callogenesis (%)	Root length	Shoot length
Smoke treatment method	4	223.333^{*}	56.667^{ns}	11.156^{***}	2.487^{***}
Error	10	40.000	66.667	0.383	0.237

** $P<0.05$; *** $P<0.001$; ^{ns} Non significant.

Table 8. The influence of different treatment methods of rapeseed MDEs with smoke-extract dilution of 1:250 on plantlet regeneration (%), root length and shoot length of regenerated plantlets. Data were analyzed after 4 weeks of culture on B_5 regeneration medium under 16:8h light/dark period at 25 ± 0.5 ^{o}C.

Smoke treatment method	Mean ± SD		
	Plantlet regeneration (%)	Root Length (cm)	Shoot Length (cm)
T_1	36.67 ± 5.78^{c}	8.67 ± 0.45^{d}	2.53 ± 0.49^{d}
T_2	40.00 ± 0^{bc}	9.37 ± 0.67^{cd}	2.93 ± 0.25^{cd}
T_3	53.33 ± 5.77^{a}	11.43 ± 0.68^{b}	3.73 ± 0.57^{bc}
T_4	50.00 ± 10.00^{ab}	10.23 ± 0.45^{c}	3.93 ± 0.46^{b}
T_5	56.67 ± 5.77^{a}	13.57 ± 0.78^{a}	4.87 ± 0.59^{a}

T_1: Control (untreated MDEs with smoke-extract solutions were transferred to B_5 regeneration medium without any smoke extract); T_2: Use of filter paper without aqueous smoke extract on top of the Petri dishes containing the B_5 medium; T_3: Inoculation of rapeseed MDEs with smoke-extract dilution of 1:250 for 15 min; T_4: Use of smoke extract (1:250) in B_5 regeneration medium; T_5: Use of filter paper saturated with aqueous smoke extract (1:250) on top of the Petri dishes containing the B_5 medium were compared to. Means with the same letter (s) are not significantly different from each other.

It was observed that use of filter paper saturated with smoke-extract dilution of 1:250 significantly improved the plantlet regeneration and growth of rapeseed MDEs compared to control and other treatment methods. Use of filter paper on top of the culture medium was previously described by Zhao et al. (1996). In study of these researchers, transferring embryos to a filter paper placed on agar medium led to increase the normal plant regeneration upto 60%, this was 1.5 times more than embryos placed directly on agar medium.

Much effort has been directed at improving the techniques for efficient plant regeneration directly from the rapeseed MDEs. Polsoni et al. (1988) reported that, when MDEs were first transferred to liquid B_5 medium with 0.1 mg l^{-1} gibberellic acid (GA_3) and 1% sucrose, and then plated on B_5 solid medium supplemented with 0.1 mg l^{-1} GA_3 and 2% sucrose for further development 10 days later, 40-60% of embryos could develop into plants directly from the primary shoot apex. Senaratna et al. (1991) reported that when embryos, at the cotyledonary stage, were treated with 50 μM ABA for 7 days before they were dried to less than 15% moisture and stored dry for at least 7 days, the conversion rates of the dry embryos into viable seedlings was about 40-50%. In a study by Zhang et al. (2006), the highest rates of plantlet development (58.46%) were obtained by exposing microspore-derived embryos to chilling at 4 ^{o}C. In the present study, rapeseed MDEs pretreated (inoculated) with smoke-extract dilutions showed a higher conversion frequency (up to 50%) compared with the control cultures (26.67%). Furthermore, application of plant-derived smoke extracts in B_5 regeneration medium enhanced the conversion frequency of rapeseed MDEs up to 62% compared with untreated MDEs (26.67%). The protocol reported in the present study was much simpler and cheaper compared with previous studies and the conversion frequency of rapeseed MDEs was also higher than previous studies. Furthermore, in this study, the percentage of callogenesis was significantly reduced in rapeseed MDEs pretreated with smoke-extract dilutions. Embryos directly converting into plantlets may minimize possible genetic variation caused by callus formation. It will be especially useful for gene transformation and crop improvement via androgenesis in *B. napus*.

Overall, hormone-like activity of plant-derived smoke extract and having an interaction with other PGRs indicate the potential that exists for the utilization of plant-derived smoke in *in vitro* systems, but it is evident that smoke extracts may also include toxic substances with detrimental effects.

Direct application of 3-methyl-2H-furo [2,3-c] pyran-2-one, instead of using smoke extract might be suggested, though it is costly. Thus, the use of smoke-water may be a feasible and inexpensive technique for plant tissue culture systems.

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