African Journal of Biotechnology Vol. 9(26), pp. 4050-4055, 28 June, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Influence of genotype and plant growth regulator on somatic embryogenesis in rapeseed (*Brassica napus* L.)

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Accepted 6 April, 2010

Two genotypes of *Brassica napus* species (Talayeh and RGS003) and the explants segment (hypocotyls and cotyledon) were tested for their potential to produce somatic embryos in *in vitro* condition. The effect of genotype, different explants and also different concentrations of plant growth regulators (PGRs) including: α-naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D), 6-benzyl amino purine (BAP), were investigated. The basal medium was Murashige and Skoog (MS) supplemented with 2% sucrose and 1% agar. Explants plated on different callus induction medium and each 4 week was subculture in the same medium. The results showed that 'Talayeh', in terms of regeneration, had significant difference with RGS003 and produce more somatic embryos. In this genotype, modified MS medium containing 2% sucrose, 6 mg l^{-1} sodium chloride (NaCl), 3 mg l^{-1} BAP, 2 mg l^{-1} NAA and 2 mg l^{-1} 2,4-D was the most efficient medium for somatic embryogenesis in 'Talayeh' and also, hypocotyl explants were found to be more suitable than cotyledon for somatic embryogenesis.

Key words: *Brassica napus* L., Talayeh, RGS003, hypocotyls, somatic embryogenesis.

INTRODUCTION

The oilseed rapes (*Brassica napus*, *Brassica rapa* and *Brassica juncea*) are now the third most important source of edible vegetable oil in the world (Burbulis, 2004). Rapeseed is one of the most important oil plants for genetic engineering (Dovzhenko, 2001). Over the last decade, researchers have made great efforts in developing biotechnological methods to facilitate rape breeding (Kott, 1998). For example, somatic embryogenesis in rapeseed has been studied extensively including the use of hypocotyls (Dwarkesh et al., 1995) and cotyledons (Turgut et al., 1998). *In vitro* culture and embryo rescue techniques are successfully done in this species (Zhang et al., 2004; Tang et al., 2003). In recent years, considerable efforts are being directed toward the improvement of important agronomic traits of plants including rapeseed through biotechnological techniques (Afolabi et al., 2004; Kahrizi et al., 2007; Hun et al., 2009). Tissue culture generates a wide range of genetic variation in plant species, which can be incorpo-rated in plant variations from different methods of tissue cultures and are also regarded as important sources for crop breeding (Jain, 2001).

The regeneration pathways of plants from somatic cell cultures have been defined as either organogenesis (Christianson et al., 1987) or somatic embryogenesis (Ammirato, 1985, 1987). Somatic embryogenesis has potential applications for both plant breeding practice and research (Burbulis et al., 2007). Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos. Somatic embryos morphologically

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Abbreviations: **PGRs**, Plant growth regulators; **NAA**, α-naphthalene acetic acid; **2,4-D, 2,4**-Dichlorophenoxy acetic acid; **BAP**, 6-benzyl amino purine; **MS**, Murashige and Skoog; **2,4-D,** dichlorophenoxy acetic acid.

resemble zygotic embryos. They are bipolar and bear typical embryonic organs; the radicle, hypocotyls and cotyledons. The greatest importance of somatic embryos is its practical application in large scale vegetative propagation (Von Arnold et al., 2002). Plant regeneration (Chen et al., 1999), somaclonal variation (Gorecka and Krzyanowska, 1997; Kirti et al., 1991), *in vitro* selection investigation (Kirti and Chopra, 1989), providing hospitallization for plant transgenic and the transfer of controller genes of desirable characteristics in plants are practical benefits of somatic embryogenesis (Ostry and Skilling, 1988).

However, including somatic embryogenesis to overcome various environmental and genetic factors that prevent fertilization is currently being focused on. Somatic embryogenesis is an efficient means of cloning because somatic embryos, unlike products of organogenesis, are believed to originate from single cells and are relatively free from gross genomic alterations (Vasil, 1987). Such clones can be selected within *in vitro* condition to tolerate abiotic and biotic stress (Narasimhulu et al.,1992). Embryogenesis can also be use for the zygotic embryogenesis studies. Gene transfer into embryogenic plant cells is already challenging conventional plant breeding and has become an indispensable tool for crop improvement (Litz and Gray, 1995). Genetic transformation is an important tool for crop improvement and genetic studies. A prerequisite of transformation for crop improvement is a high rate of regeneration from tissue culture. Efficient plant regeneration methods would facilitate genetic transformation of commercial barley cultivars (Jha et al., 2007). According to Kayim and Koc (2006), protoplast fusion technologies rely on embryogenic callus culture and such technologies are often used for genetic improvement.

Furthermore, embryogenic cultures or somatic embryos are amenable to *in vitro* cryo-preservation and this could provide a long-term storage solution for seeds. The objective of the study was to identify efficient culture growing conditions for mass multiplication of *B. napus* through somatic embryogenesis. There are many factors which play a vital role in somatic embryogenesis and these include organic acids and vitamins (Pullman et al., 2006). The response of embryos to different culture medium formulations could depend on the plant species. Somatic embryogenesis is a more efficient pathway for studies involving production of genetically transformed plants (Litz and Gray, 1995). Although obtaining the embryo of rapeseed micro spores culture has been carried out by researchers, in somatic embryogenesis of vegetative organ explants, research is very little (Majd et al., 2006). In this study, an attempt is made to find out effects of different genotypes, explants and also different concentrations of plant growth regulators (PGRs) including auxin: α-naphthalene acetic acid (NAA), 2,4- dichlorophenoxy acetic acid (2,4-D), cytokinin 6-benzylaminopurine (BAP) on somatic embryogenesis in rapeseed.

MATERIALS AND METHODS

Plant material

This study was conducted in The Biotechnology and Molecular Genetics Lab, Zanjan University, during year 2008 - 2009. The rapeseeds (*B. napes* cvs. Talayeh and RGS003) was provided by Iranian Seed and Plant Improvement Institute. Initially, mature rapeseeds were incubated in 20°C for 30 min and then were surface sterilized using 70% alcohol and commercial hypochlorite sodium solution (20%) for 20 s and 15 min, respectively, and rinsed 4 - 5 times with sterile distilled water.

Medium for seed culture

Rape seeds were placed on half strength MS (Mourashige and Skoog, 1962) medium with 10 g l^{-1} agar, 20 g l^{-1} sucrose without any PGR. The pH of the medium was adjusted to 5.8 before adding agar, then autoclaved at 121°C for 15 min. The cultures were grown at 25±1°C, under white fluorescent tubes with 16/8 h photoperiod at 200 µmol m^{-2} s^{-1}.

Production of embryogenic callus

One week after rape seed germination, segments measuring 5 - 7 mm length from hypocotyls, and 2 mm area from cotyledon were prepared and placed on callus induction and regeneration medium. This medium (callus induction and regeneration) was modified MS medium containing 10 g l^{-1} agar and 20 g l^{-1} sucrose with 6 g l^{-1} sodium chloride (NaCl). The concentrations of PGRs were: 2,4-dichlorophenoxy acetic acid (2,4-D) (1 and 2 mg l^{-1}); NAA (1, 2 and 3 mg l^{-1}); BAP (1, 2 and 3 mg l^{-1}).

Explants were cultured into 80 mm diameter sterile plastic Petri dish with 5 explants per each. They were then kept in darkness conditions for 15 days to produce callus and embryogenesis. In order to develop embryos, the culture was kept at 25±1°C temperature, 16/8 h photoperiod under white fluorescent tubes for 2 months. Observation was done every day, and features such as callusing, somatic embryos, organogenesis and some disorders (browning, infesting) were monitored. Average number of maturity morphologic embryos per explants was recorded 30 days after being transferred to light conditions. To determine the percentage of the embryos for germination, the embryos at cotyledonary stage, were separated into single embryos and transferred onto the culture medium (without any PGRs).

Statistical analysis

Tests were set up on factorial experiment in completely randomized design (CRD) form three replications. Observations per explants were recorded. Data were transformed to satisfy the normality assumptions using ($\sqrt{X}$) formula. Analysis of variance was performed to evaluate the main effects and interaction between genotypes, explants type and hormone level, then Duncan's test and T test were performed (α =0.05). Data analysis using the MSTATC software was evaluated.

RESULTS AND DISCUSSION

The results of this study revealed that the embryogenesis

Table 1. The results of analysis of variance according to the analyzed number of somatic embryo (F value).

Source	DF	Mean Square
Factor A	1	130.247**
Factor B	1	19.052**
AB	1	19.326**
Factor C	17	2.820*
AC	17	2.193^{ns}
BC	17	0.739
ABC	17	0.956
Error	144	1.655

Factor A: Genotype; factor B: explants; factor C: plant growth regulators; ** Significant at α = 0.01; *Significant at α = 0.05; ns: non significant.

of *B. napus* L. produced on basal medium supplemented with different concentration of plant growth regulators was significantly influenced by genotype and concentrations of PGRs. Of the two genotypes, 'Talayeh' and 'RGS003', 'Talayeh' was more suitable for regeneration and embryogenesis. Of the two plant sources, hypocotyls explant was more appropriate than cotyledon for somatic embryogenesis in both gene types. Statistical analysis show that, in the terms of regeneration, there was a significant difference (α = 0.01) between cotyledon and hypocotyl of the two genotypes (Table 1). Explants produced from hypocotyls segment were healthy and vigorous when compared with other explants. The rate of explant elongation during the first week culture was enhanced by darkness conditions (data are not shown). Furthermore, explants began to swell after one week of initial culture in all induction media and callus formed on the cut edge of cotyledon and hypocotyls explants. Different stages of embryogenesis are shown in Figure 1. At the 15^{th} day of explants culture, most calluses were cream in colour. The first part that started callus induction and had most induction, was cotyledon. Considering that the callus induction and regeneration medium were the same, callus growth was limited. After 15 days, when the explants were transferred to light condition, callus growth was almost stopped and after a few days (about 20-30 days after explants culture) embryo induction started. The mechanisms that control cell differentiation during somatic embryogenesis are far from being clear. However, secreted, soluble signal molecules play an important role (Van Arnold et al., 2002). Embryos that usually appear at the end is cut and generally, their numbers is low (3 – 12 per explant). Embryogenesis in the dark lighting cycle occurred much more in the once darkness (Narasimhulu et al., 1992). Primary somatic embryos produced on MS basal medium with the same concentrations of PGRs, were maintained by sub culturing on the same medium. The important point on callus was that not all embryos in same stage of development and different stages of development simultaneously, had the same result (Natalija et al., 2004; Majd et al., 2006; Narasimhulu et al., 1992). This can be due to differences in hormonal concentration gradient used. The functional mechanism of dark treatment in promoting plant regeneration is not clear. First, incubation in the dark may delay degradation of endogenous or exogenous plant growth regulators (BAP, 2,4-D and NAA) which trigger and direct plant regeneration *in vitro* (Ahn et al., 2007). Secondly, as reported by Herman and Hess (1963), dark treatment may reduce the levels of cell wall thickness and cell wall deposits (cellulose and hemicelluloses), facilitating translocation of plant growth regulators in plant cells. Light may be an important means of controlling secondary embryogenesis in *B. napus* (Shu and Loh, 1987). Embryo incubation in continuous darkness or continuous light led to a marked suppression of secondary embryogenesis. The best light regime for secondary embryogenesis was 16 h light/8 h dark. Loh et al. (1983) speculated that endogenous plant growth regulators might be involved in the initiation of secondary embryogenesis in *B. napus.*

The secondary embryogenic potential of *B. napus* L. could be maintained for many years on complete MS medium without diminution (Loh and Lim, 1992). Shu and Loh (1987) reported that secondary embryogenic potential of winter oilseed rape was maintained for over six years without diminution. Synthetic auxins (NAA and 2,4-D) are usually used for initiating embryogenic cultures. One mechanism whereby auxins may regulate embryogenesis is through acidification of the cytoplasm and cell-wall (Kutschera, 1994). Most commonly used is 2,4-D at a concentration of 1 to 10 µM. However, in some cases it is essential to combine different auxins (NAA). Initiation of embryogenic cultures by cytokinin (BAP) alone is relatively rare, but for many species (rapeseed), it is important to combine auxin and cytokinin (Majd et al., 2006). Loh and Lim (1992) found that NAA increase embryogenesis. Similarly, 2,4-D increased embryogenesis, but weakly (Narasimhulu et al., 1992). Table 2 shows, application of 3 mg l^{-1} BAP, and 3 mg l^{-1} NAA plus 1 mg l^{-1} 2,4-D, which resulted in the greatest embryogenesis in RGS003 genotype. Whereas, for 'Tallayeh' genotype, the best results were obtained on basal MS medium containing 3 mg l^{-1} BAP, 2 mg l^{-1} NAA and 2 mg l^{-1} 2,4-D (Table 3). Our results disagree with Majd et al. (2006), who reported that on MS medium supplemented with 2 mgl^{-1} BAP, 2 mg l^{-1} NAA and 1 mg l^{-1} 2,4-D, hypocotyls produced somatic embryos.

On the whole, it can be concluded that 'Talayeh' is the best genotype for regeneration when compared with RGS003 genotype, and also hypocotyls explants were found to be more suitable than cotyledon for somatic embryogenesis. Koh and Loh (2000) stated that created cuts on the explants released phenolic components, which caused embryogenesis induction on the explants, therefore hypocotyls with two cuts at the end, showed healthy

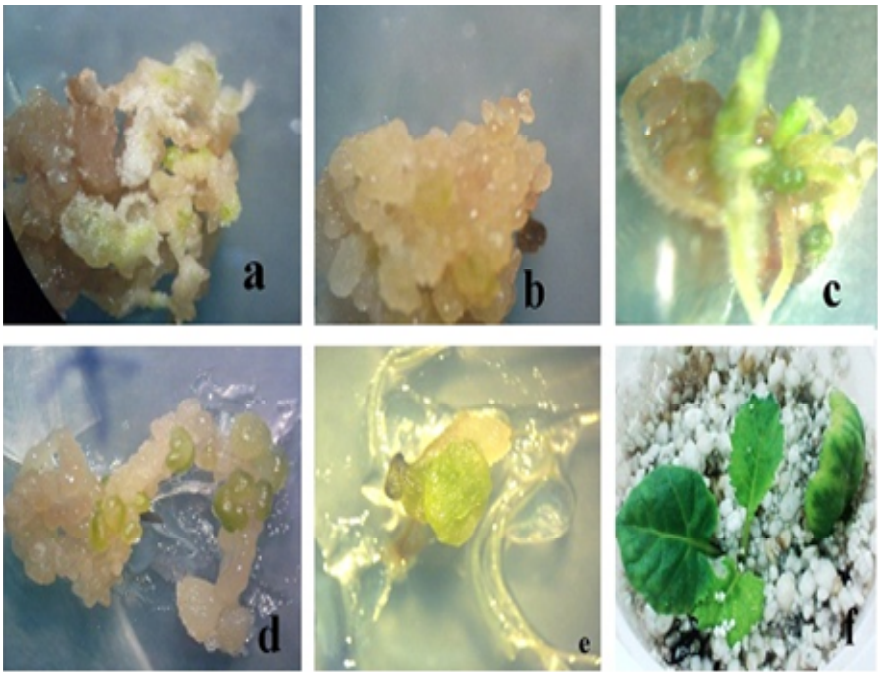

Figure 1. Different stages of embryogenesis of *Brassica napus* cv. Talayeh on MS medium. a. embryo in globular stage(after 6 weeks); b. heart shape stage on cotyledon callus; c. torpedo stage on cotyledon callus; d. cotyledonary embryo on hypocotyls callus; e. root formation of explant; f. one week after transfer of plantlet to soil.

Table 2. Effect of different concentrations of plant growth regulators (BAP, NAA, 2,4-D) and explants segment (hypocotyls and cotyledon) on somatic embryogenesis in *Brassica napus* cv. RGS003.

Treatment(mg l^{-1})			Average number of somatic embryos per explants	
BAP	**NAA**	**2,4-D**	**Hypocotyls**	**Cotyledon**
1	1	1	1.73^{a}	1.60^{abcd}
1	1	2	1.38^{abc}	1^{de}
1	2	1	0.7^{d}	1.03^{de}
1	2	2	0.8^{cd}	0.9^{e}
1	3	1	1.51^{ab}	0.93^{e}
1	3	2	1.48^{ab}	0.93^{e}
2	1	1	1.66^{a}	1.24^{bcde}
2	1	2	1.27^{abcd}	1.82^{ab}
2	2	1	0.9^{bcd}	1.13^{cde}
2	2	2	1.60^{a}	1.98^{a}
2	3	1	1.73^{a}	1.38^{abcde}
2	3	2	1.14^{abcd}	1.33^{abcde}
3	1	1	1.28^{abcd}	1.03^{de}
3	1	2	1.38^{abc}	0.9^{e}
3	2	1	1.17^{abcd}	1.60^{abcd}
3	2	2	1.13^{abcd}	1.48^{abcde}
3	3	1	1.68^{a}	1.96^{a}
3	3	2	1.51^{ab}	1.71^{abc}

Data were collected after 2 month of culture on MS basal medium supplemented with the indicated growth regulators. Values are the means of at least three replicates; means followed by different letters are significantly different (P = 0.05).

Table 3. Effect of different concentrations of plant growth regulators (BAP, NAA, 2,4-D) and explants segment (hypocotyl and cotyledon) on somatic embryogenesis in *Brassica napus* cv. Talayeh.

Treatment (mg l^{-1})			Average number of somatic embryos per explants	
BAP	**NAA**	**2,4-D**	**Hypocotyls**	**cotyledon**
1	1	1	3.66^{efg}	5.33^{defg}
1	1	2	5.33^{def}	3.66^{ghij}
1	2	1	3.66^{efgh}	3^{ij}
1	2	2	2.33^{h}	2.66^{j}
1	3	1	3.33^{fgh}	3.33^{hij}
1	3	2	4.33^{efg}	5.33^{defg}
2	1	1	3^{gh}	5.33^{ghij}
2	1	2	5.66^{cde}	6.33^{cdef}
2	2	1	5.33^{def}	3^{ghij}
2	2	2	3.66^{efgh}	7.33^{bcde}
2	3	1	3.66^{efgh}	5.66^{defg}
2	3	2	3.33^{fgh}	4.66^{fghi}
3	1	1	8.33^{bc}	11.33^{a}
3	1	2	7.66^{bcd}	9^{abc}
3	2	1	9.33^{b}	6.33^{def}
3	2	2	12.33^{a}	9.33^{ab}
3	3	1	8.33^{b}	5^{efgh}
3	3	2	7^{bcd}	7.66^{bcd}

Data were collected after 2 month of culture on MS basal medium supplement with the indicated growth regulators. Values are the means of at least three replicates; means followed by different letters are significantly different (P = 0.05).

growth.

It is obvious that embryogenesis was genotype dependent, 'Talayeh' produced more embryos than 'RGS003' (Tables 2 and 3). The main reason for the obtained results is probably due to the fact that the effects of genotype impose one of the greatest constraints on plant tissue regeneration. In some instances, observed responses have been related by identifiable genetic factors: nuclear genes (Szakcs et al., 1988; Mathias and Fukui, 1986; Lall et al., 2004), cytoplasmic genes (Narasimhulu et al., 1988; Wan et al., 1988) and gene interactions (Wan et al., 1988).

Unfortunately, the behavior of plant tissue regeneration (embryogenesis) *in vitro* processes such as callus formation, growth and regeneration, often seem to be under an over-riding genetic control (genotype), with other factors exerting only a minor effect. The genetic control of embryogenesis *in vitro* can result from either a primary or secondary effect of gene action. Such genes in specific genotype have been thought most likely to exert their influence by regulating the effective levels of growth substances (Bayliss and Dunn, 1979). Endogenous growth substance levels may vary in different genotypes.

ACKNOWLEDGEMENTS

Special thanks to the Plant Biotechnology Laboratory of the Zanjan University for their generous help and to Mir. Azimkhani for providing a considerable valuable data.

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