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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (3): 481-485, 2007
ISSN 1028-8880

Comparison of Somatic Embryogenesis in *Medicago sativa* and *Medicago truncatula*

F. Hoori, A.A. Ehsanpour and A. Mostajeran
Department of Biology, Faculty of Science, Isfahan University, Isfahan, Iran

Abstract: In this study, the regeneration through embryogenesis of two species of *Medicago* were studied. Seeds of *Medicago sativa* cv. Rehnani and *M. truncatula* line A17 were grown on MS medium. After 4-6 weeks, segments of leaf and stem from two species were transferred to MS medium containing 2 mg L^{-1} NAA, 2,4-D and Kinetin. The results indicated that callus formation from leaf explants of *M. sativa* was higher than *M. trancatula*. In the next stage, media with different combinations of auxin, cytokinin or ethinyl estradiol were provided for regeneration. Then in two stages, explants of leaf and stem of two species were transferred on these media. Results after 3-6 weeks showed that in medium containing NAA and TDZ, stem pieces of *M. sativa* produced shoots while leaf pieces on NAA and ethinyl estradiol formed roots. Leaf explants of *M. truncatula* in the medium containing NAA and BAP, produced somatic embryos. Also in media with auxin and ethinyl estradiol, somatic embryos were formed on calli of two species. Ethinyl estradiol and auxin together can induce somatic embryogenesis and root production on calli and stem or leaf explants.

Key words: *Medicago sativa*, *Medicago truncatula*, callus, regeneration

INTRODUCTION

Medicago truncatula Gaertn. (known as barrel medic because of the shape of its seeds pods) is an annual species (Fabaceae) that is native to the Mediterranean area and is widely grown as a pasture legume and crop rotation in a number of regions throughout the world (Crawford *et al*., 1989). This species is an autogamous diploid species (2n = 2X = 16) with a small genome (Neves *et al*., 1999). This plant species is self fertile and has short life cycle of about 3 months (Barker *et al*., 1990; Araujo *et al*., 2004). These characteristics enables this species to be used in molecular genetic studies like analysis of gene expression, promoter functional analysis, T-DNA mutagenesis and expression of genes for crop improvement (Somers *et al*., 2003). It is also a model plant for the study of legume-rhizobium symbiosis (Barker *et al*., 1990).

Medicago sativa L. is not annual species but is closely related to *M. truncatula*. This species has high degree of heterozygosity and large genome size (2n = 32) (Iantcheva *et al*., 1999).

Legumes have been regarded as recalcitrant to transformation and their *in vitro* regeneration is highly genotype dependent and only rarely cultivated varieties are amenable to regeneration (Somers *et al*., 2003).

Regeneration through somatic embryogenesis is frequently of single cell origin, resulting in a low frequency of chimera and a high number of regenerants (Neves *et al*., 1999). These characteristics render somatic embryogenesis an attractive system for the introduction of gnomic traits of interest by genetic engineering (Neves *et al*., 1999). Usually an auxin is required to induce somatic embryogenesis and subsequent auxin withdrawal or lowering of the auxin concentration is required for embryo maturation (Dudtis *et al*., 1991; Nolan *et al*., 2003; Von Arnold *et al*., 2002). Auxin play critical roles in the major growth responses during plant development. At cellular level, auxin acts as a signal for division, expansion and differentiation throughout the plant life cycle. By contrast less attention has been directed to effect of steroids on plants. In addition to the hormone regime, key variables are explant, developmental stage, nutrition regime and genotype (Rose and Nolan, 1995). Regeneration via organogenesis or embryogenesis are the basis of tissue culture methods and without regeneration, it is impossible to produce transgenic plants.

In this study, we considered the effect of several growth regulators including ethinyl estradiol on somatic embryogenesis of *M. truncatula* line A17 and *M. sativa* cv. Rehnani.

MATERIALS AND METHODS

Seeds of *M. truncatula* line A17 were obtained from State Agriculture and Biotechnology Center (SABC) WA. Seeds of *M. sativa* cv. Rehnani were supplied from Seed and Seedling Research Center of Isfahan. Seeds of

Corresponding Author: A.A. Ehsanpour, Department of Biology, Faculty of Science, Isfahan University, Isfahan, Iran

Table 1: MS medium with plant growth regulators for plant regeneration

R1	NAA(0.5 mg L^{-1})+ Ethinyl estradiol (0.5 mg L^{-1})
R2	BAP(0.9 mg L^{-1})+ NAA (1.8 mg L^{-1})
R3	IAA(0.1 mg L^{-1})+ Ethinyl estradiol (1 mg L^{-1})
R4	IAA(0.1 mg L^{-1}) + BAP (1 mg L^{-1})
R5	NAA(0.5 mg L^{-1})+ TDZ (0.5 mg L^{-1})
R6	Ethinyl estradiol (0.5 mg L^{-1})
R7	NAA(0.5 mg L^{-1})
C	NAA + 2,4-D + Kinetin (2 mg L^{-1})

M. truncatula first scarified by sands for germination. Then seeds of both species were surface sterilized for 30 sec with ethanol (70% v/v) followed by 30% (v/v) sodium hypochlorite for 15 min. After 3-4 rinses by sterile distilled water, seeds of *M. sativa* were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 1% (w/v) agar while, seeds of *M. truncatula* first were cultured on 1% (w/v) water agar medium and after two weeks seedlings were transferred to MS medium with the same combination as described already. The pH of all media was adjusted to 5.8 before autoclaving (15 min at 121°C).

For callus induction, leaf and stem segments of *in vitro* grown seedlings were transferred to callus proliferation, C medium.

Cultures were maintained in the culture room with 16/8 light-dark photoperiod at 25±2°C. The experiments were carried out with 10 replications and 4 explants in each replication. In the next stage, after two subcultures calli were transferred to MS medium supplemented with different growth regulators according to Table 1.

Embryogenic calli then were transferred to hormone free medium to develop somatic embryos.

All data were analyzed according to Duncan or Chi-Square tests using SPSS and Sigma Stat programs.

RESULTS

Stem and leaf explants of *M. sativa* and *M. truncatula* on C medium produced high percentage of soft calli as Table 2 showing fresh weight of callus resulted from stem is relatively lower than leaf.

When calli obtained from either stem or leaf were transferred to 7 different media for somatic embryogenesis, a few media induced somatic embryos in both *Medicago* species. For instance, leaf and stem callus produced the highest percentage of embryos in R1 medium (Table 3), while, in *Medicago truncatula* leaf and stem callus produced the highest percentage of somatic embryo in R3 and R1 media, respectively (Table 4). In other media with different combination of plant growth regulators we observed different percentage of somatic embryo initiation within 20-30 days after calli were transferred to the embryogenesis media. Development of somatic embryos from leaf segments of *M. sativa* and *M. truncatula* are illustrated in Fig. 1.

In R5 medium, calli obtained from stems and leaves of *M. sativa* grew very well and became green and after 35 days formed shoot buds. In this medium, leaf

Table 2: Callus production in C medium. Uncommon letters are significant ($p<0.05$)

C medium	Explant	Callus induction (%)	Fresh weight (g)
M. sativa	Leaf	100	2.41^{a}
	Stem	100	1.751^{b}
M. truncatula	Leaf	88	0.637^{c}
	Stem	100	0.45^{c}

Table 3: The effects of different combination of growth regulators on somatic embryogenesis of *Medicago sativa* callus

Explant	Medium	% of somatic embryo
Leaf callus	R1	63
″	R2	0
″	R3	50
″	R4	46
″	R5	0
″	R6	0
″	R7	0
Stem callus	R1	80
″	R2	0
″	R3	0
″	R4	42
″	R5	0
″	R6	0
″	R7	0

Table 4: The effects of different combination of growth regulators on somatic embryogenesis of *Medicago truncatula* callus

Explant	Medium	% of somatic embryo
Leaf callus		
″	R1	67
″	R2	0
″	R3	80
″	R4	58
″	R5	63
″	R6	17
″	R7	29
Stem callus	R1	79
″	R2	0
″	R3	42
″	R4	0
″	R5	50
″	R6	0
″	R7	58

Table 5: Comparing the number of somatic embryos formed per callus of stems and leaves in *M. sativa* and *M. truncatula* in different media based on Chi-Square analysis on 5% (comparisons are columnar). Uncommon letters are significant ($p<0.05$)

M. sativa			*M. truncatula*		
Medium	Leaf callus	Stem callus	Medium	Leaf callus	Stem callus
R1	2.5^{a}	2.2^{a}	R1	2.5^{a}	2.5^{a}
R2	0.0	0.0	R2	0.0	0.0
R3	2.5^{a}	0.0	R3	3.1^{b}	2.2^{b}
R4	2.0^{b}	2.0^{b}	R4	2.7^{c}	0.0
R5	0.0	0.0	R5	2.0^{d}	3.0^{e}
R6	0.0	0.0	R6	1.7^{f}	0.0
R7	0.0	0.0	R7	2.7^{e}	1.4^{d}

Uncommon letter (s) are significant

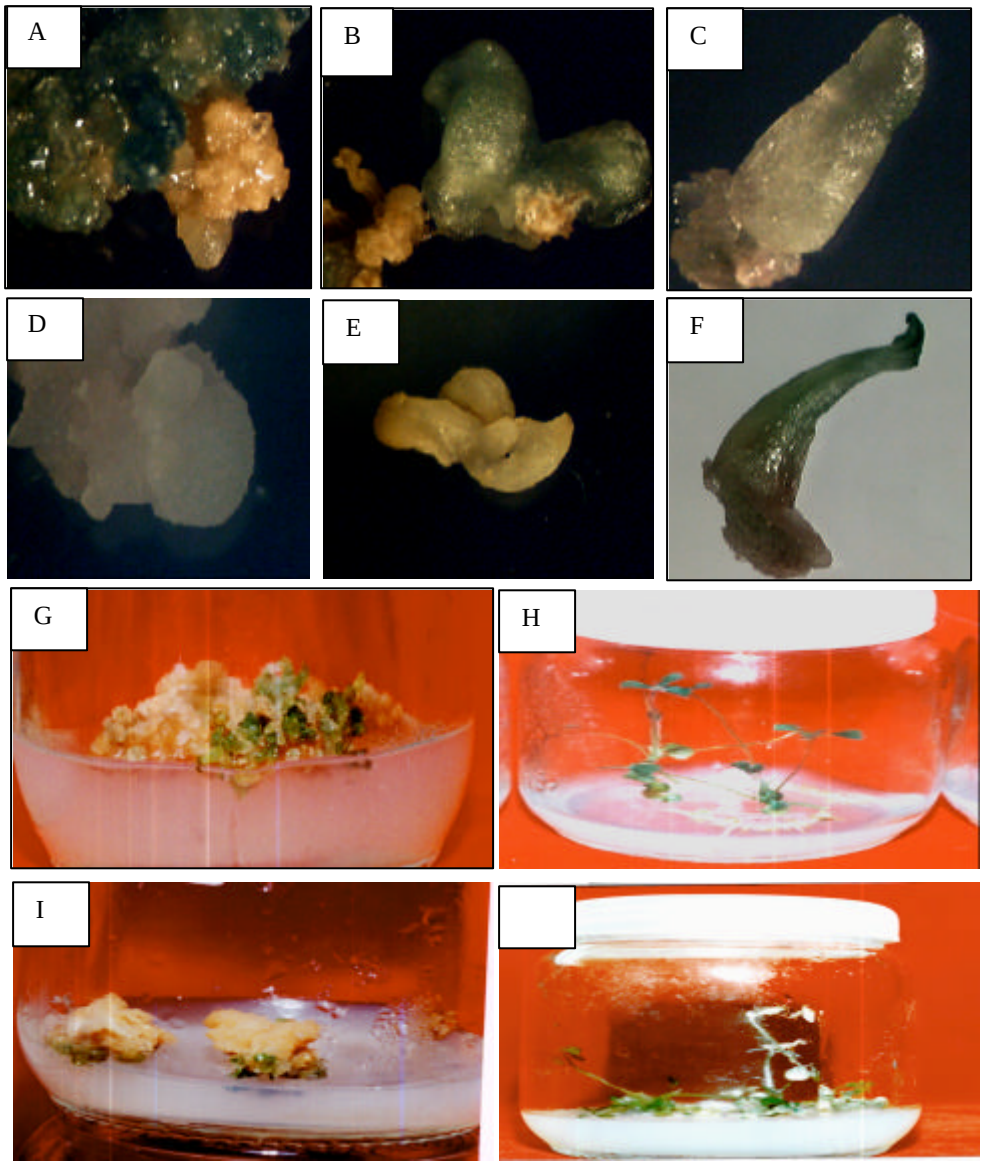

Fig. 1: Different stages of somatic embryo development From *M. sativa* (A, B, C, G, H), *M. truncatula* (D, E, F, I, J), letters are: globular stage, heart stage, torpedo stage, plant let formation, mature plant, respectively

segments produced green calli but no shoots were developed. Comparing of somatic embryogenesis data for both *Medicago* species in different media are illustrated in Table 5.

DISCUSSION

We present here an efficient procedure for somatic embryogenesis using a suitable combination of plant growth regulators in particular using ethanol estradiol for *Medicago trucatula* line A17 and *Medicago sativa* cv. Rehnani. At the first step, calli were formed on MS medium containing 2 mg L^{-1} of each three growth regulators (NAA, 2,4-D and kinetin). In respect to R7 medium (NAA 0.5 mg L^{-1}), we concluded that NAA alone is not enough for callus formation but combination of auxin and cytokinin and their possible interaction in the medium promote callus formation. Our results agree with Saunders and Bingham (1972). They reported that B-II basal medium supplemented with 2 mg L^{-1} of NAA, 2,4-D and kinetin produced calli from anther, cotyledons, hypocotyls and internodes culture of *M. sativa* within 4 weeks. Nolan and Rose (1998) has also showed that NAA alone can not induce callus formation in *M. truncatula*. It

has been well documented that 2,4-D (a strong growth regulator) with combination of cytokinin and yeast extract as a source of organic nitrate stimulates callus formation and somatic embryogenesis in *M. sativa* (McKersie and Brown, 1997, Saunders and Bingham, 1972). We found that this combination of plant hormones in MS medium promote callus formation from *M. truncatula*.

The responses of various explants to the medium is depending to the type of explant. For example, leaves were more responsive explants for dedifferentiation and callus formation in *Medicago sativa* while, stems produced more callus than leaves in *M. truncatula*.

Our data showed that NAA or ethinyl estradiol alone could not form callus but rather induced root formation. Estradiol is the strongest form of natural estrogen that resembles in central nucleus to cholesterol and is synthesized from testosterone (Rawn, 1989). Ethinyl estradiol may have auxin-like activity that its combination with NAA strengthened its effect and cause root formation from leaf explants of *M. sativa*.

In R2 medium, 4.2% of leaf explants of *M. truncatula* developed plantlets via indirect regeneration. It has been shown that NAA and BAP in the medium are very effective for induction of somatic embryogenesis of *M. truncatula* (Nolan and Rose, 1998; Nolan *et al*., 1989).

McKersie and Brown (1997) reported that IAA is not an effective auxin in callus formation and induction of somatic embryogenesis in *M. sativa*. Application of ethinyl estradiol alone (R6 medium) had no effects on embryo development. Therefore, combination of IAA and ethinyl estradiol (R3) had better effect on embryo formation.

Explants of leaves and stems of *M. sativa* in R5 medium formed calli but only stem pieces produced shoots. The auxin/cytokinin ratio is an important factor for regeneration. Increasing of cytokinin level may lead to shoot formation. Our results indicated that in R1 medium root and embryogenic masses formation may occur from interaction of auxin and ethinyl estradiol in this medium. According to some datas, auxin acts synergistically with estradiol (Goda *et al*., 2004; Bajguz and Tretyn, 2003). Cytokinin-like activity of ethinyl estradiol in combination with auxin might be another reason for embryo formation (Bao *et al*., 2004).

TDZ is a synthetic compound which is a derivative of phenylurea and has been reported to possess strong cytokinin-like activity in a number of plants (Huetteman and Preece, 1993). It has been used successfully for shoot regeneration in diverse plant species including woody plants and leguminous plants such as beans and peanut (Matand *et al*., 1994). However, presence of TDZ probably prevented somatic embryogenesis in calli derived from leaves and stems of *M. sativa* and *M. trancatula*.

Our data are in contrast with Iantcheva *et al*. (1999), they proposed that high frequency of somatic embryogenesis induced by TDZ might influence the endogenous level of cytokinins, auxins and abscisic acid so as induce the positive embryogenic response of cultivated tissue.

It can be concluded that embryogenic potential of many *Medicago* species is genotype specific. In addition, type of explant, its genetic potential, combination of plant growth regulators in the medium and endogenous level of hormone in tissues are the most effective factors on somatic embryogenesis and plant regeneration.

ACKNOWLEDGMENT

Authors thank the Graduate Directorate of Isfahan University for their support of this project.

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