J. Plant Biotechnology(2004) Vol. 6(3). pp. 171~179

Journal of Plant Biotechnology

Hormonal Requirements Induced Different Regeneration Pathways in *Alhagi graecorum*

A. M. Hassanein*

Genetics Lab. Faculty of Science, South Valley University, 82524 Sohag, Egypt.

Abstract

Hormonal requirements inducing different regeneration pathways with particular emphasis on somatic embryogenesis in *Alhagi graecorum* were studied. While combination of 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5 μM 6-benzylaminopurine (BAP) and 5 μM 1-naphthaleneacetic acid (NAA) in MS medium induced callus formation and callus maintenance from internodal explants, each alone or in combination with other induced distinct regeneration pathway. Adventitious bud formation was induced on MS medium supplemented with 2.5 μM BAP. It was improved when 2.5 μM BAP was used in combination with 5 μM NAA. MS medium containing 0.5 μM 2,4-D or 5 μM NAA induced the formation of abnormal direct somatic embryos. While increase of 2,4-D concentration (1.125- 9) resulted in the formation of viable embryogenic mass, increase of NAA did not change its effect. NAA should be used in combination with 2,4-D even at low concentration (0.5 μM) to form embryogenic mass. In *A. gaecorum*, the role of 2,4-D as trigger of somatic embryogenesis and BAP as trigger of adventitious bud formation was deduced, but for maximum yield certain auxin-cytokinin ratio should be applied. Embryogenic masses characterized by high water content, low peroxidase activity, and low number of peroxidase and glutamate oxaloacetate transaminase bands in comparison with calli obtained under conditions stimulating adventitious bud formation. The resulted differential gene expression, which could be detected by native-PAGE patterns, could be used as marker for organogenic pathway in *A. graecorum*.

* Corresponding author, E-mail: hassaneinam@yahoo.com
Received Apr. 15, 2004; Accepted Aug. 25, 2004

Key words: Growth regulators, isoenzymes, organogenesis, somatic embryogenesis

Introduction

Alhagi graecorum Bioss is a wild plant belonging to family Fabaceae. It grows in xeric, halic and mesic habitats (Boulos and El-Hadidi 1984; El-Khatib et al. 1999). In the desert, camels and goats graze it. Dry plants are used as a laxative and vermifuge for bilharziasis and rheumatic pains (Boulos and El-Hadidi 1984). *A. graecorum* had shown interesting aspects with regards to regenerate plants from young and mature materials, especially when tiny-mature explant method was used (Hassanein and Mazen 2001). The stress tolerance and high regeneration ability of *A. graecorum* may be used to improve Fabaceae species via protoplast fusion. It depends on two facts: (1) the regeneration ability is a dominant trait (Harms 1983) and it is very high in *A. graecorum* (Hassanein and Mazen 2001), and (2) somatic hybridization offers advantages over sexual hybridization particularly in facilitating wilder crosses (Rose et al. 1990).

By modifying the *in vitro* culture conditions a distinct morphogenic program can be induced (Endress 1994; De Klerk et al. 1997; Mezzetti et al. 1997). In tissue culture, there are two basic morphogenic programs: somatic embryogenesis and organogenesis, however, few comparisons have been made between them within individual plant. The type of regeneration pathway could be predicted based on the callus phenotype. Plant growth regulators have profound effects in this concern due to their role in regulation of gene expression (Hare et al. 1996; Hassanein et al. 1999a,b). Studies on organogenesis has revealed that various factors affect plant development, in addition to

phytohormones. But how such factors act on cultured tissue, to induce organogenesis, still need further studies (Ozawa et al. 1998). Physical and chemical composition of medium, explant type, species, and water status of the cultured tissue affect the specific capacity of the tissue to regenerate (Etienne et al. 1991; Endress 1994; Carron et al. 1995; Hassanein and Mazen 2001; Hassanein et al. 2003). A few specific proteins and isoenzyme forms associated with embryogenesis and organogenesis were reported (Fellers et al. 1997; Hassanein et al. 1999a,b). Biochemical and molecular basis as well as the phenotypical characteristics of calli following different regeneration pathways have not been extensively studied. In this work, phenotype, water content, peroxidase activity, native-PAGE and isoenzyme patterns of calli, under the influence of different phytohormones, were studied to detect a useful marker to determine early the morphogenic pathways of the cultured tissue. To our knowledge, somatic embryogenesis of *A. graecorum* has not been extensively studied. Therefore, particular emphasis on somatic embryogenesis was aimed. Since all types of explants, isolated from young or mature tissue, showed organogenic potential of shoot formation (Hassanein and Mazen 2001), their embryogenic potential was also studied.

Materials and Methods

Plant materials

Alhagi graecorum seeds were surface sterilized by dipping in 5% Clorox solution for 5 min followed by 5 min dip in 75% ethanol. The seeds were rinsed three times in sterile water, 5 min each. The seeds were germinated on MS (Murashige and Skoog 1962) medium supplemented with 2.5 μM BA. After 7 days, the seedlings were used as a source of explants to induce different morphogenic pathways. Shoot culture was established by subculturing shoot cutting of seedlings on basal MS medium to be a source of internodal explants. Moreover, newly emerged shoots of *A. graecorum* plants grown naturally in very dry area were cut, sterilized and used as a source for nodal and internodal explants.

Culture conditions

Unless otherwise stated, the following conditions applied: cultures were maintained under white fluorescent tubes, providing illumination of 60 μM m^{-2} s^{-1}, in growth room at 25℃ ± 2 with 16 h daily photoperiod. Internodal explants (obtained from shoot culture) were used to induce different morphogenic pathways under the influence of growth regulators. Media for organogenesis or embryogenesis experiments comprised MS medium supplemented with 30 g/L sucrose and 0.8% agar (w/v), pH 5.8 (prior to autoclaving for 20 min at 121℃). The vitamin part was (expressed as mg/L): myoinositol (100), Vitamin B1 hydrochloride (4), nicotinic acid (4), pyridoxal hydrochloride (0.7), biotin (0.04) and folic acid (0.5). The growth regulators were added prior to adjusting pH and autoclaving. Media were dispensed into 90 mm diameter Petri dishes or 250 ml jars.

All the experiments were repeated thrice, in each 45 explants were cultured in three Petri dishes (11 cm diameter). All data were subjected to analysis of variance using statistical package. The significant difference of the average was tested using Least Significance Differences (L. S. D) at 5% significant level.

Effect of growth regulators on regeneration pathways

Where required, MS medium supplemented with one or more of the following growth regulators: 2.5 μM BA, 5 μM NAA and 0.5 μM 2,4-D (Table 2) was used to induce different morphogenic pathways on internodal explants. After three weeks, the texture of growth on the internodal explants was examined; the f. wt, d.wt and percent of water content (pwc) were calculated. After 4 weeks, the percentage of explants showing embryogenic mass, adventitious buds and somatic embryos was counted. To improve somatic embryogenesis, different concentrations of 2,4-D (1.125- 9 μM) or NAA (5 and 6.5 μM) were used. Also, 4.5 μM 2,4-D in combination with different concentrations of (Kinitin) KIN or BAP (1.25- 3.75 μM) were investigated (Table 2).

Effect of explant type on somatic embryogenesis

To test the effect of explant type on somatic embryogenesis, different seedling explants (cotyledons, hypocotyl, epicotyl and roots of 7 days old seedling), stems of *in vitro* grown shoots (internodal, nodal and leaf segments) and wild grown plants (nodal and internodal) were cultured on MS medium supplemented with 4.5 μM 2,4-D. After 4 weeks, the embryogenic masses were transferred to MS medium supplemented with 1.25 μM NAA for embryo maturation. The number of embryos was determined. Mature embryos were transferred to MS medium supplemented with 5 μM IBA and 1 mg/L vitamin D3 to get plantlets.

Electrophoretis analysis and relative peroxidase activity

In two weeks, calli formed under the influence of 2.5 μM BAP, 5 μM NAA and 0.5 μM 2,4-D (conditions stimulated formation and maintenance of undifferentiated calli) as well as those formed under the influence of 2.5 M BAP (condition stimulated adventitious bud formation) or 4.5 μM 2,4-D (condition stimulated embryogenic mass formation) were used for native-PAGE and isoenzyme analysis. Half gram of calli was ground at 4℃ in a mortar in 0.5 ml of extraction buffer that consisted of 0.1 μM tris-HCl, pH 7.0, and 0.002 M cysteine. The homogenate was centrifuged at 15000 g at 4℃ for 15 min. Supernatants were collected for immediate electrophoresis in 7.5% polyacrylamide slab gels. Gels were run at 18 mA for 6 h at 10℃ in 0.025 M tris + 0.192 M glycine buffer (pH 8.9). Two isoenzymes were stained: peroxidase (Siegel and Galston 1967) and glutamate *oxaloacetate transaminase* (Brewer 1970).

For native-PAGE (polyacrylamide gel electrophoresis) analysis, samples containing equal amounts of protein were loaded. The gel was stained in 0.2% coomassie Brilliant Blue Q25 in methanol, acetic acid and water (30: 10: 60 v/v), destained in the same solution without dye until the protein bands were visualized. The Rf value was determined from the mean of observations resulted from five independent electrophoretic runs of five separate extractions as described by Bertozo and Valls (2001). The protein bands were separated on the bases of their size, shape and net charge (Gepts 1990).

Estimation of relative peroxidase activity was measured as described by Wakamatsu and Takahama (1993). The extracted samples were assayed spectrophotometrically at 470 nm. The assay mixture for peroxidase activity contained 40 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 5 mM guaiacol, and 0.3 mM hydrogen peroxide. The peroxidase activity of calli formed on MS medium supplemented with 2.5 μM BA + 5 μM NAA + 0.5 μM 2,4-D was considered to be a control (100%).

Results and Discussion

Effect of culture conditions on water content of the explant

Since *A. graecorum* grows in halic, xeric and mesic habitats, it can control the efficiency of metabolic pathways, which are needed to establish sufficient cell turgor and consequently *in vitro* culture. The pwc of wild grown plants was relatively low (75.98%). Segments of these plants showed an increase in pwc upon their transfer to MS medium, but it was still lower than that of established shoot culture (Table 1). The high pwc of tissue culture materials may be due to stimulation of water absorption, which may be due to the chemical composition of the medium, and reduction of water loss by culture container. Increased cell turgidity and consequently cell activity were essential prerequisite for morphological processes (Stafford and Warren 1993).

Table 1. Water content of wild plant in comparison to that of segments of wild plants cultured *in vitro*. Means ± standard deviation of three independent experiments. 45 explants were used for each experiment. * Significant differences in relation to the corresponding reference control.

Plant materials	% water content
Shoot of wild grown plants (Control)	75.98 ± 0.80
Shoot of wild grown plants, after sterilization	82.66 ± 0.82*
Segments of wild grown plants cultured on MS medium for one day	81.11 ± 0.52*
Segments of wild grown plants cultured on MS medium for 3 days	86.06 ± 0.95*
Shoot culture	90.73 ± 0.41*

Characteristics of calli initiated under the influence of different hormonal treatments to induce different regeneration pathways

Morphogenesis processes can be determined into organogenesis or embryogenesis and each pathway, needed a distinct hormonal requirement. Growth regulators' combination induced callus formation and callus maintenance, but they induced different morphogenic pathways when used individually or in combination (Table 2). Complex interaction takes place during morphogenesis between external and internal phytohormones determining cell competence (Mezzetti et al. 1997). In three weeks, under all determined conditions, the tissues of *A. graecorum* commenced and continued cell division to form calli. It was accompanied by increasing the pwc (Table 2) especially when the cells were induced to follow the embryogenic pathway and formed mucilaginous growth (96.23%). In general, the development phase led the somatic embryos on callus to the cotyledonary stage accompanied with high turgor pressure and relative pwc (Etienne et al. 1991). Microscopic investigation showed that somatic embryos consisted of extremely differentiated vacuolized cells (Carron et al. 1995). Water status appears to be a crucial parameter for *in vitro* culture especially via somatic embryogenesis pathway (Etienne et al. 1991).

Also, data in Table 2 shows that the highest fresh mass

Table 2. The effect of different growth regulators on morphogenetis pathway, growth (fresh and dry masses) and water content. ADF- adventitious bud formation; EMS- embryogenic mass; DSE- direct somatic embryogenesis; AD-adventitious buds; SE- somatic embryos.
Means ± standard deviation of three independent experiments. 45 explants were used for each one
* Significant differences in relation to the corresponding reference control.

KIN μM	BAB μM	NAA μM	2,4-D μM	Morpho. percentage	Type of morpho.	Fresh weight/explant	Dry weight/explant	Water content (%)	Number of SE or AD/explant	Number of plantlets/ explant
- Control	2.5	5	0.5	100	Callus	0.480 ± 0.11	0.037 ± 0.008	92.29 ± 0.13	-	-
-	2.5	5	-	100	ADF	1.032 ± 0.25*	0.080 ± 0.011*	92.02 ± 0.30	50 ± 3.06 AD	35 ± 4.00
-	-	5	0.5	100	EMS	0.817 ± 0.37*	0.047 ± 0.002	94.61 ± 0.27	10 ± 1.15 SE	4 ± 1.53
-	2.5	-	0.5	40 ± 4.51	DSE	0.515 ± 0.10	0.043 ± 0.006	91.67 ± 1.20	6 ± 1.53 SE	0.0
-	2.5	-	-	100	ADF	0.415 ± 0.11	0.035 ± 0.008	91.75 ± 0.20	16 ± 3.06 AD	11 ± 3.0
-	-	5	-	36 ± 3.06	DSE	0.563 ± 0.14	0.038 ± 0.009	93.13 ± 0.16*	2 ± 0.58 SE	0.0
-	-	6.5	-	35 ± 4.33	DSE	0.518 ± 0.06	0.032 ± 0.009	93.82 ±0.31*	1 ± 0.58 SE	0.0
-	-	-	0.5	26 ± 5.58	DSE	0.502 ± 0.06	0.031 ± 0.009	93.19 ± 0.93*	1 ± 0.58 SE	0.0
-	-	-	1.125	100	EMS	0.892 ± 0.08*	0.040 ± 0.011	95.52 ± 0.48*	6 ± 1.00 SE	2 ± 0.00
-	-	-	2.25	100	EMS	1.032 ± 0.07*	0.05 ± 0.002	95.16 ± 0.20*	17 ± 2.08 SE	8 ± 1.15
-	-	-	4.5	100	EMS	1.120 ± 0.31*	0.041 ± 0.003	96.23 ± 0.79*	15 ± 2.0 SE	8 ± 1.00
-	-	-	9.0	100	EMS	1.085 ± 0.04*	0.048 ± 0.004	95.58 ± 0.31*	13 ± 1.53 SE	6 ± 1.15
-	2.5	-	4.5	100	EMS	1.203 ± 0.09*	0.062 ± 0.003*	94.85 ± 0.21*	20 ± 3.06 SE	11 ± 1.53
1.25	-	-	4.5	100	EMS	1.065 ± 0.07*	0.052 ± 0.003	95.08 ± 0.30*	21 ± 1.15 SE	8 ± 1.00
2.5	-	-	4.5	100	EMS	1.213 ± 0.08*	0.051 ± 0.002	96.00 ± 0.22*	23 ± 2.0 SE	11 ± 3.21
3.75	-	-	4.5	100	EMS	1.060 ± 0.05*	0.052 ± 0.003	95.06 ± 0.09*	15 ± 4.2 SE	6 ± 02.65

(f.wt) of calli was obtained under suitable conditions of somatic embryogenesis when they were initiated on MS medium supplemented with 2.5 μM BAP and 4.5 μM 2,4-D. On the other hand the highest dry mass (d.wt) was obtained when the calli were initiated under suitable conditions of adventitious bud formation on MS medium containing 2.5 μM BAP and 5 μM NAA.

All the tested growth regulators and their combinations allowed callus formation. By the third week, four distinct types of calli were initiated. First type: internodal explants cultured on MS medium supplemented with 2.5 μM BAP, 5 μM NAA and 0.5 μM 2,4-D (MTH medium) formed whitish-compact calli (Figure 1A), they were maintained for more than one year by monthly subculturing on MTH medium. De Klerk et al. (1997) reported that formation of callus may facilitate the response of cultured tissues to regenerate via organogenesis or embryogenesis upon their transfer to the medium containing the proper stimulus. In *A. graecorum* the response to the application of phytohormones was high on explant or after callus formation where MS medium containing 2.5 μM BAP induced shoot formation on explant as well as on callus. The appearance of callus initiated on MTH medium was little changed when subcultured on shoot formation medium. In MS medium supplemented with 2.5 M BAP, it became more brown at the basal side, more green on the upper side than callus grown on MTH (Figure 1C). On the other side, when the calli were transferred to medium supplemented with 4.5 μM 2,4-D, translucent creamy growth commenced on the basal side of the callus to form embryogenic mass. Second type: greenish yellow compact callus was initiated on internodal segments cultured on MS medium containing 2.5 μM BAP. The lower side of these calli turned brown. It was clear that addition of 5 μM NAA with 2.5 μM BAP improved the formation of adventitious buds and accompanied with the appearance of slight brown color at lower side of calli. As detected in *A. graecorum*, the onset of tissue browning, before the concentration of phenolic compounds become high, did not cause a loss of viability but led to vigorous growth and metabolism with increased organogenesis (Lindfors et al. 1990). By the fourth week, the calli showed adventitious bud formation (Figure 1C). Kim et al. (2001) reported that both auxin and cytokinin coordinately regulated the shoot formation. Third type: translucent creamy yellow calli were initiated on internodal segments cultured on MS medium containing 0.5 μM 2,4-D and 5 μM NAA. This type of growth was better on MS medium containing high concentration of 2,4-D alone (Figure 1B) or in combination with cytokinins. Calli produced on medium supplemented with 4.5 μM 2,4-D and 2.5 μM BAP tended to be more yellowish in color, grew more quickly, produced lesser pigment and produced higher number of somatic embryos than other growth regulator combinations. They were maintained for more than 4 months by monthly subculture onto new medium. Kim and Kim (2002) reported that among tested auxins, 2,4-D was the most suitable for friable callus production. Fourth type: direct somatic embryos were observed in four weeks when the calli were initiated from internodal explants cultured on MS medium supplemented with 5 μM NAA or low concentration of 2,4-D (0.5 μM). The radicle of embryos grew outside the callus (Figure 1D). The somatic embryos could be separated (Figure 1F) and cultured individually on basal MS medium, but rarely showed further development to form plantlets. It was only possible when they were initiated on MS medium supplemented with 0.5 μM 2,4-D. Somatic embryogenesis may be

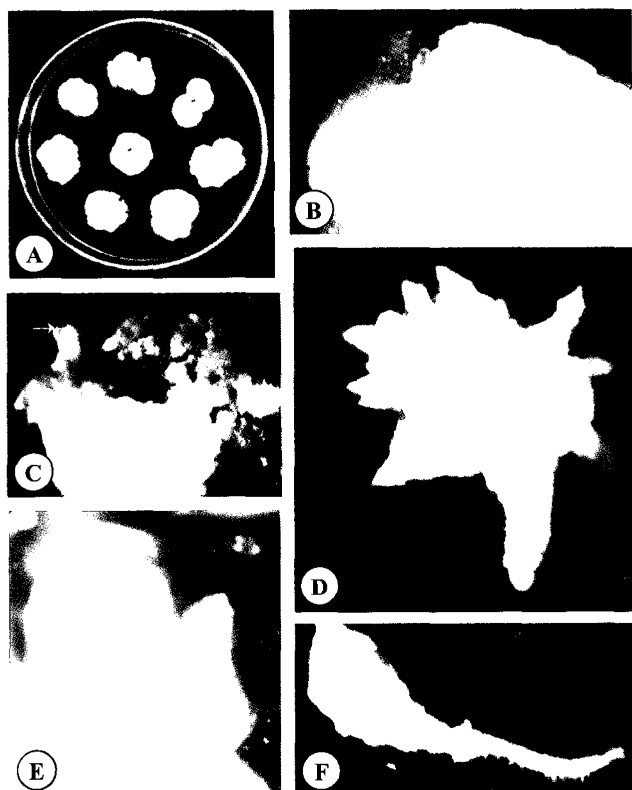

Figure 1. Different regeneration pathways of *A. graecorum* under the influence of different growth regulators; A) Calli induced from internodal explant on MS medium supplemented with 0.5 μM 2,4-D, 2.5 μM BAP and 5 μM NAA, B) Embryogenic mass induced from nodal explant on MS medium supplemented with 4.5 μM 2,4-D, C) Regeneration via adventitious bud formation on callus (obtained on MS medium supplemented with 2.5 μM BA+ 5 μM NAA+ 0.5 μM 2,4-D) subcultured on MS medium supplemented with 2.5 μM BAP, D) Direct somatic embryogenesis induced from internodal explant cultured on MS medium supplemented with 0.5 μM 2,4-D, E) Mature somatic embryos resulted from embryogenic mass on MS medium supplemented with 1.125 μM NAA, F) Direct somatic embryo after its separation from embryo aggregate (D).

blocked or may be abnormal due to factors such as artificial hormonal levels or interactions that are a product of the tissue culture itself (Vasil 1982).

The presence of 2,4-D in relatively high concentration, e.g. 4.5 μM 24-D, was a prerequisite for SEs initiation and consequently for their transformation to complete plants. The presence of auxin NAA at concentration of 5 μM was important when the 2,4-D was in low concentration (0.5 M). Calli produced on MS medium supplemented with 5 μM NAA and low concentration of 2,4-D (0.5 μM) grew slower and produced more pigment but lesser mucilage than calli produced on medium containing higher concentrations of 2,4-D alone or in combinations with cytokinins. This negative correlation between pigment production and formation of friable embryogenic calli was reported in other plant species (Kaeppler and Pedersen 1996).

In this work, 0.5 μM 2,4-D induced the formation of direct somatic embryogenesis, but increase in its concentration (1.125- 9) resulted in the viable embryogenic mass (Table 2). On the other side, 5 μM NAA stimulated the direct somatic embryogenesis and increase in its concentration more than 5 μM NAA did not change its effect. It was clear that addition of 2,4-D even at low concentration (0.5 μM) was an essential prerequisite to form embryogenic mass. Therefore, the role of 2,4-D as trigger of somatic embryogenesis was deduced, but for maximum yield of SEs certain auxin-cytokinin ratio should be applied. Consequently, addition of 2.5 μM BAP or KIN to MS medium containing 4.5 μM 2,4-D resulted in improving SEs yields and addition of 5 μM NAA to MS medium containing 2.5 μM BAP improving the yield of adventitious buds.

Study of gene expression in *A. graecorum* tissues accompanied different regeneration pathways following different hormonal treatments

In 4 weeks, the compact calli showed brown colour on the lower side, especially on callus showing adventitious bud formation on MS medium supplemented with 2.5 μM BAP. Callus consistency and colour was an indicator of the organogenesis capacity of callus (Schween and Schwenkel 2003). The translucent calli never showed brown colour during the first culture (4 weeks) or after their transfer to MS medium supplemented with 1.125 μM NAA until the appearance of SEs in 2 months (Figure 1E). It seems that browning results from low water content of the tissue, where as mucilaginous tissues are characterized by high water potential and high water content (Carron et al. 1995). Close relationship between callus embryogenic potential and a specific water status has been demonstrated (Eteinne et al. 1991). Since peroxidase activity is affected by the water content of the tissue, consequently, the peroxidase activity of compact tissue should be higher than mucilaginous tissues (Table 3). Maximum peroxidase activity and a modification of isoperoxidases were noted at the same time during the formation of adventitious buds in various plant species (Chawla 1991; Hassanein et al. 1999a,b, Arezki et al. 2001).

Increasing the staining intensity of POX bands (Figure 2A) under conditions of stimulated adventitious bud formation in comparison to that stimulated embryogenic mass formation gives additional indication about increase of the peroxidase activity prior to adventitious bud formation. In addition to increase in the staining intensity of POX bands, two new POX bands (POX-1 and POX-8) were only detected during the tendency of tissue to adventitious bud formation, which could be used as a marker of organogenesis in *A. graecorum*. Increase in peroxidsae activity automatically accentuated auxin catabolism, resulting in lowering endogenous auxin level. Consequently, an optimal auxin/ cytokinin ratio favors the formation of adventitious buds (Legrand and Bouazza 1991).

Conditions stimulating somatic embryogenesis expressed the lowest number of peroxidase bands (Figure 2A) and relative peroxidase activity; it is in accordance with results obtained by Wakamatsu and Takahama (1993). It may be due to the nature of these calli, where they were characterized by high turger pressure and low osmotic potential, which are favorable characteristics for somatic embryogenesis (Carron et al. 1995). As in case of peroxidase, expression of glutamate oxaloacetate transaminase bands (Figure 2B) of calli formed under suitable conditions for adventitious bud formation was higher than those formed under conditions of somatic embryogenesis. In the latter, two bands (GOT2 and GOT3) were not detected. Varying expression of GOT and POX according to tissue and developmental stage of the tissue was reported by several authors (Visedo et al. 1990; Protopapadakis and Papanikolaou 1998; Hassanein 1998; Hassanein et al. 1999b)

The native protein patterns of calli formed in two weeks

Table 3. The effect of regeneration pathways on relative peroxidase activity of internodal explants cultured for 2 weeks on media induced callus formation, adventitious bud formation or somatic embryogenesis. Mean of 5 independent experiments was used to calculate the relative peroxidase activity in relation to the corresponding reference control (100%).

Regeneration pathway	Relative peroxidase activity
Callus (control)	100
Adventitious buds	170
Somatic embryogenesis	37

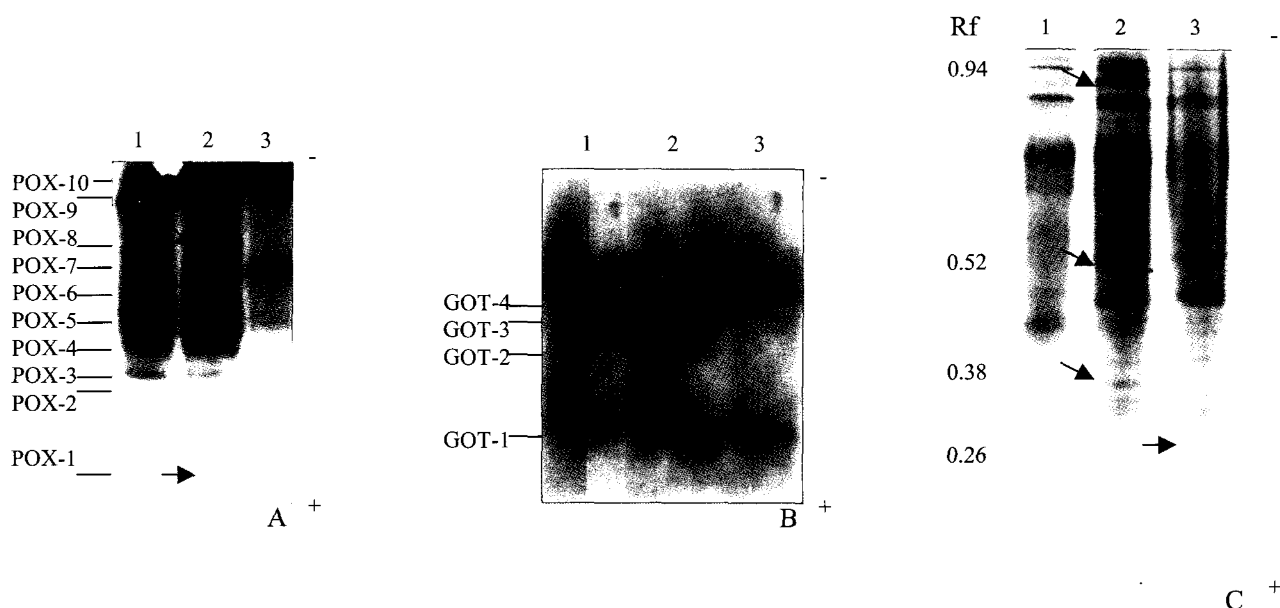

Figure 2. Peroxidase (A), *glutamate oxaloacetate transaminase* (B) *and native*-PAGE (C) of calli initiated in two weeks on internodal segments to follow different morphogenic pathways; Lane (1)- internodal segment cultured on MS medium supplemented with 2.5 μM BAP, 5 μM NAA and 0.5 μM 2,4-D to form and maintain callus; Lane (2)- internodal segments cultured on MS medium supplemented with 2.5 μM BAP to form adventitious buds; Lane (3)- internodal segments cultured on MS medium supplemented with 4.5 μM 2,4-D to form embryogenic mass.

culture under different conditions stimulating different regeneration pathways are represented in Figure 2C. In comparison to the isoenzyme patterns, high number of bands in the native-PAGE increased the chance to find specific variation for each regeneration pathway. In this concern, while three additional bands (Rf-0.94, Rf-0.52 and Rf-0.38) were detected under conditions stimulating shoot organogenesis only one faint band with Rf of 0.26 was detected under conditions stimulating somatic embryogenesis. Proteins specific to embryogenic growth have been reported in other plant species (Fellers et al. 1997). The detected differential gene expression may be resulting from the induced regeneration pathway irrespective of the inducer used. The patterns of isoenzymes and native-PAGE was the same if the embryogenesis was initiated by 2,4-D alone, or in combination with other growth regulators (data not shown), or where the organogenesis was initiated by BAP alone or in combination with NAA. Studies on somatic embryogenesis indicated that 2,4-D does not directly affect genes controlling somatic embryogenesis but it does have strong effect in the development of somatic embryos (Bokird et al. 1988).

Effect of the source of explant on somatic embryogenesis efficiency

The major requirements for obtaining effective explant tissue are high cell division potential and morphogenic plasticity. The most interesting feature of *A. graecorum* was its competence to respond to *in vitro* culture conditions irrespective the source of the explant. Regions of actively dividing cells responded most readily, therefore, explants derived from seedling or shoot culture showed a high response under defined culture conditions. Seedling and shoot cultures were preferred as explant sources to induce somatic embryogenesis in this species and other systems (Cuenca et al. 1999). Also, *A. graecorum* explants derived from mature wild plants were used successfully, but produced less number of SEs than those of shoot culture or seedling materials (Table 4). The uptake of hormones by explants from the medium may be different (Peeters et al. 1991), which differentially affects the metabolism of endogenous hormones (Li and Neumann 1985). *A. graecorum* is similar to *Daucus carota*, since any part of the plant body was successfully used to obtain somatic embryos (Ammirato, 1983). In the previous studies on *A. graecorum* (Hassanein and Mazen 2001), all explants derived from tissues near the cotyledons of seedlings showed high tendency to initiate adventitious buds. In the present work, the effect of exlpant position to the cotyledons on the somatic embryogenesis showed less straightforward relationship. The internodal segments were preferred to fulfil all the experiment in this work because they were the easiest explant to obtain.

Table 4. Number of embryos obtained from different types of explants cultured on MS medium supplemented with 4.5 μM 2,4-D. Means ± standard deviation of three independent experiments. 45 explants were used for each experiment.
* Significant differences in relation to the corresponding reference control.

Source of explant	Number of embryos
Seedling	
Cotyledon (Control)	25 ± 2.00
Hypocotyl	22 ± 1.00
Epicotyl	22 ± 0.58
Shoot culture	
Node	22 ± 0.58
Internode	23 ± 1.15
Leaf	15 ± 2.52*
Root	20 ± 2.08*
Wild grown plants	
Node	12 ± 2.52*
Internode	13 ± 3.79*

Somatic embryos maturation

For somatic embryogenesis to occur, the embryogenic mass formation must be given the essential prerequisite to continue its program initiated during the first culture (embryo initiation and multiplication). Embryogenic tissue of rapidly dividing cells (4 weeks old) was transferred for further development (embryo maturation) on medium supplemented with 1.125 μM NAA for 2 months. It is well known that medium supplemented with low concentration of auxin induces embryogenesis from tissues, which are already embryogenically determined (Ammirato 1983; Blanc et al. 1999). Stafford and Warren (1993) reported that the usual trigger for somatic embryogenesis is the removal of auxin, or the use of less potent auxin e.g. NAA. The mature SEs were germinated on MS medium supplemented with 5 μM IBA and 1 mg/L vitamin D3.

References

Ammirato PV (1983) Embryogenesis. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (ed), Handbook of Plant Cell Culture, Vol 1, pp 82-123. Mac Millan, New York

Arezki A, Boxus P, Kevers C, Caspar T (2001) Changes in peroxidase activity, and level of phenolic compounds during light-induced plantlet regeneration from *Eucalyptus camaladulensis* Dehn. Nodes *in vitro.* Plant growth Regul 33: 215-219

Bertozo MR, Valls JFM (2001) Seed storage protein electophoresis in *Arachis pintoi* and *A. repens* (Leguminosae) for evaluating genetic diversity. Genetic Resour Crop Evol 48: 121-130

Blanc G, Michaux-Ferriere N, Teisson C, Lardet L, Carron MP (1999) Effect of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasliensis.* Plant Cell Tiss Org Cult 59: 103-112

Borkird C, Choi JH, Jin Z, Franz G, Hatzopolous P, Chorneau R, Bonas U, Pelegri F, Sung ZR (1988) Development regulation of embryonic genes in plants. Proc Natl Acad Sci USA 85: 6399-6403

Boulos L, El-Hadidi M (1984) The Weed Flora of Egypt. The American Univ., Cairo, Egypt

Brewer GJ (1970) Introduction to isoenzyme techniques. Academic Press, New York, San Francisco, London

Carman JG (1990) Embryogenic cells in plant tissue cultures occurrence and behavior. *In vitro* Cell Dev Biol 26: 746-753

Carron MP, Etienne H, Michaux-Ferriere N, Montoro P (1995) Somatic embryogenesis in rubber tree (*Hevea brasiliensis* Mull. Arg.) In: Bajaj YPS (ed.) Biotechnology in Agriculture and Forestry. Vol 30. Somatic Embryogenesis and Synthetic Seed. pp 353-369. Springer Verlag Berlin.

Chawla HS (1991) Plant regeneration potential and isoenzyme variation during morphogenesis of barley callus. Biol Plant 33: 175-180

Cuenca B, San-Jose MC, Martinez MT, Ballester A, Vieitez AM (1999) Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Rep 18: 538-543

De Klerk GJ, Arnholdt-Schmitt B, Lieberei R (1997) Regeneration of roots, shoots and embryos: physiological biochemical and molecular aspects. Biol Plant 39: 53-66

El-Khatib AA, Fayez KA, Hassanein AM (1999) Adaptive responses of *Alhagi graecorum* under different habitat conditions. Acta Agronomica Hungarica 47: 171-180

Endress R (1994) Plant Cell Biotechnology. Springer Verlag, Hiedelberg

Etienne H, Berger A, Carron MP (1991) Water status of callus from *Hevea brasiliensis* during induction of somatic embryogenesis. Physiol Plant 82: 213-218

Fellers JP, Guenzi AC, Porter DR (1997) Marker proteins associated with somatic embryogenesis of wheat callus cultures. J Plant Physiol 151: 201-208

Gepts P (1990) Genetic diversity of seed storage proteins in plants. In: Brown HD, Glegg MT, Kahler AL and Weir BS (eds), Plant Population, Genetics, Breeding and Genetic Recourses, pp 64-82. Sunderland, Massachusetts

Hare PD, du Plessis S, Cress WA, Van Staden J (1996) Stress induced changes in plant gene expression. South African J Sci 92: 431-439

Harms CT (1983) Somatic incompatibility in the development of higher plant somatic hybrids. The Quarterly Review of Biology 58: 325-353

Hassanein AM (1998) Isoezyme patterns of *Solanum nigrum* and the cybrid plant containing *Solanum nigrum* genome

and *Solanum tuberosum* plastome. Biol Plant 40: 617- 621

Hassanein AM, Mazen AMA (2001) Adventitious bud formation in *Alhagi graecorum*. Plant Cell, Tiss Org Cult 65: 31-35

Hassanein AM, Ahmed AM, Abdel-Hafez AII, Soltan DM (1999a) Phenol-oxidizing isoenzymes, malate dehyderogenase patterns and organogenesis of *Solanum nigrum* as affected by light treatments. Acta Agrono Hungar 47: 127-136

Hassanein AM, Ahmed AM, Abdel-Hafez AII, Soltan DM (1999b) Isoenzymes expression during root and shoot organogenesis of *Solanum nigrum*. Biol Plant 42: 341-347

Hassanein AM, Galal AA, Azooz MM (2003) Interaction between time of nodal explant collection and growth regulators determines the efficiency of *Morus alba* microprobagation. J Plant Biotech 5: 255-231

Kaeppler HF, Pedersen JF (1996) Media effects on phenotype of callus cultures initiated from photoperiod-insensitive, elite inbred sorghum lines. Maydica 41: 83-89

Kim SH, Kim SK (2002) Effects of auxins and cytokinins on callus induction from leaf blade, petiole, and stem segments of *in vitro*-grown 'Sheridan' grape shoots. J Plant Biotech 4: 17-21

Kim KH, Park HK, Yeo UD (2001) Effect of auxins and cytokinins on organogenesis of soybean *Glycine max* L. J Plant Biotech 3: 95-100

Legrand B, Bouazza A (1991) Changes in peroxidase and IAA-oxidase activities during adventitious buds formation from small root explant of *Cichorium intybus* L.: Influence of glucose. J Plant Physiol 138: 102-106

Lindfors A, Kuusela H, Hohtola A, Kupila-Ahvenniemi S (1990) Molecular correlates of tissue browning and deterioration in Scots pine calli. Biologia Plantarum 32: 171-180

Li T, Neumann KH (1985) Embryogenesis and endogenous hormone of cell culture of some carrot varieties (*Daucus carota* L.). Berl Deut Bot Ges 98: 227-235

Mezzetti B, Savini G, Carnevali F, Mott D (1997) Plant genotype and growth regulators interaction affecting *in vitro* morphogenesis of blackberry and raspberry. Biol Plant 39: 139-150

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

Ozawa S, Yasutani I, Fukuda H, Komamine A, Sugiyama M (1998) Organogenic Reponses in tissue culture of mutants of *Arabidopsis thaliana*. Development 125: 135-142

Peeters AJM, Gerads W, Barendse GWM (1991) *In vitro* flower bud formation in tobacco: interaction of hormones. Plant Physiol 97: 402-408

Rose RJ, Thomas MR, Fitter JT (1990) The transfer of cytoplasmic and molecular genomes by somatic hybridization. Aust J Plant Physiol 17: 303-321

Protopapadakis E, Papanikolaou X (1998) A study on glutamate oxaloacetate transaminase isozymes of citron cultivars. Genetic Resour Crop Evol 45: 561-564

Schween G, Schwenkel H (2003) Effect of genotype on callus induction, shoot organogenesis, and phenotypic stability of regenerated plants in the greenhouse of *Primula* ssp. Plant Cell Tiss Org Cult 72: 53-61

Siegel BZ, Galston AW (1967) The isoperoxidase of *Pisum sativum*. Plant Physiol 42: 221-226

Stafford A, Warren G (1993) Plant Cell and Tissue, John Willy & Sons, Chichester, New York

Wakamatsu K, Takahama U (1993) Changes in peroxidase activity and in peroxidase isoenzyme in carrot callus. Physiol Plant 88: 167-171

Vasil IK (1982) Plant Cell culture and somatic cell genetics of cereals and grasses. Vasil IK, Scowcroft W, Frew KJ (ed.s), Plant Improvement and Somatic Cell Genetics, pp 179-203. Academic Press. New York

Visedo G, Fernandez-Piqueras J, Garcia JA (1990) Isoenzyme profiles associated with the hypersensitive response of Chenopodium foetidum to plum pox virus infection. Physiol Plant 78: 218-224