Somatic embryogenesis in Allium sativum L. (cv. Yamuna Safed 3): Improving embryo maturation and germination with PGRs and carbohydrates

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Resumen

Embriogénesis somática en Allium sativum *L. (cv. Yamuna Safed 3): Mejora de la maduración del embrión y la germinación con PGRs y carbohidratos.*

A partir de la parte basal de diente de ajo, se indujo un callo en medio nutritivo Murashige & Skoog (MS), formándose embriones somáticos. En *Allium sativum* L. cv. Yamuna Safed 3, se ha dividido la embriogenia somática en tres etapas: inicio y proliferación del embrión; maduración; y germinación del embrión/conversión en plántula. El inicio de la diferenciación del estado de embrión globular se observó a varias concentraciones de BAP y 2,4-D, siendo la la máxima proliferación de embriones con 1,0 mg/l de BAP y 0,25 mg/l de 2,4-D. La mejor maduración embrionaria se obtuvo con la adicción de 0,5 mg/l GA_3; una concentración similar de ABA fue menos efectiva. El máximo crecimiento embrionario se produjo con la adición de maltosa al 3%, mientras que la máxima germinación (65,15%) se obtuvo con fructosa al 3%. Se incluyen imágenes de microscopio electrónico de barrido (SEM) para mostrar la morfología y estructura del embrión. Se tuvo éxito en la obtención de plántulas a partir de embriones desarrollados *in vitro*.

Palabras clave: *Allium sativum* L., Callo, Fuentes de Carbono, Reguladores de Crecimiento, Embrión somático.

Abstract

Callus induction was obtained from basal part of the garlic clove on Murashige and Skoog (MS) medium on which somatic embryos were formed. In *Allium sativum* L. cv. Yamuna Safed 3, somatic embryogeny has been categorized into three distinct stages: initiation and proliferation of embryo; maturation; and germination of embryo. The initiation of globular-stage embryo differentiation was observed on various BAP and 2,4-D concentrations but at 1.0 mg/l BAP and 0.25 mg/l 2,4-D maximum proliferation of embryos was noted. Best somatic embryo maturation was achieved on 0.5 mg/l GA_3 amended medium; ABA at the same level was less effective. Embryo growth was maximum on 3% maltose while plantlet conversion was highest (65.15%) on 3% fructose added medium. Scanning electron microscopy (SEM) has been presented to demonstrate the apparent morphology and structural details of embryo. Plantlets were successfully raised from *in vitro* developed embryos.

Key words: *Allium sativum* L., Callus, Carbon Sources, Plant Growth Regulators, Somatic Embryos.

Introduction

Garlic (*Allium sativum* L.) is an important and widely cultivated plant used as a food flavoring agent and it also has several medicinal applications. The active compounds show antibiotic, antitumour, cholesterol-lowering, and antithrombic effects (Milner 2001). In agriculture, garlic is used as bactericide and fungicide compound (Tokit et al. 2003). It is exclusively propagated vegetatively, a process that has low coefficient of multiplication, which demonstrates a potential risk of transmission of viral diseases (Novak 1990). As the propagation rate is low (approx. 5-10% per year) it is time-consuming and a laborious process. Therefore, it takes many years to produce sufficient number of seed bulbs for practical cultivation of a new variety (Nagakubo et al. 1993). The crop improvement by cross fertilization is limited as garlic is a sterile plant (Masanori et al. 1995). Various cultural techniques have been reported to improve the efficiency of propagation but all seem to have their own inherent reservations (Nagakubo et al. 1993) as long-term cultivation requirement, the necessity of mastering skillful techniques and poor propagation rates at times are more common.

In recent times, the application of biotechnology techniques has been used for various practical purposes including transgenics development. The transgenic processes improve crops by adding traits / genes to superior elite genotypes with a relatively short period of time. In order to be more effective, an efficient and stable plant regeneration system is essential and a primary consideration. Somatic embryogenesis offers this opportunity and thus has been exploited in a wide range of plant groups ((Rommens et al. 2004, Walter 2004). It has several advantages over organogenesis and appears to be the most promising method for large scale clonal propagation of selected elite genotypes (Ignacimuthu 1996, Lelu-Walter et al. 2008). In addition, embryogenic cells or suspensions can effectively be exploited to conserve genetic resources by *in vitro* cryopreservation. Although a few reports on embryogenesis in *Allium* are available lately (Fereol et al. 2002, Tokit et al. 2003), the synchronous proliferation of embryos, the maturation, and efficient germination are proving difficult and the information discussing those events are still not adequate. The basic understandings of the nature of somatic embryo quality that behaves like zygotic embryos and the study involving cultural conditions, which facilitate quality *in vitro* embryo production are not enough to exploit embryogeny for practical exploitation. In this present communication, we discussed various stages of embryogenesis in *A. sativum* L. cv. Yamuna Safed 3 from basal clove part. The embryo initiation and proliferation, maturation and regeneration of plantlets have been described; the role of Plant Growth Regulators (PGR) and carbohydrates has also been evaluated. The established protocol may be very useful for the production of *Allium* transgenics en masse.

Materials and methods

Plant material and explant preparation

Local Indian garlic (*Allium sativum* L. cv. Yamuna safed 3), a moderate maturing variety was used. It was procured from National Horticultural Research and Development Foundation, Nasik (Maharashtra). The cultivar of the plant was identified and voucher specimen (IC-375119) was deposited at National Bureau of Plant Genetic Resources, New Delhi, India. Bulbs were harvested from 4-5 months old plants, were stored at 15°C, and the cloves of the same source were used as explant.

Healthy garlic cloves were selected and washed under running water for 1 h, surface sterilized with 0.1% $HgCl_2$ for 15 min, 70% ethanol for 30 sec and finally washed several times with sterilized distilled water. Garlic clove was divided into basal, middle and tip part (3-5mm), later were cultured as explants.

Medium and culture conditions

The Murashige and Skoog medium (1962) (MS) supplemented with various concentrations of 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) were used. All the medium components were added and the pH 5.6 was adjusted prior to sterilization at 121ºC, for 20 min. All the cultures were incubated at 25 ± 2ºC in a culture room under 16 h photoperiod provided by cool white fluorescent light at irradiation of 40 $Wm^{-2} S^{-1}$.

Initiation of embryogenic callus

Basal part of the clove (3-5 mm) was excised

aseptically and cultured on MS which was added with 3% sucrose, 100 mg/l inositol, 2.0 mg/l 2,4-D and 0.5 mg/l BAP. The same medium without PGRs was also tested for the induction of embryogenic callus. The presence or absence of early stages of somatic embryos was characterized by small, dense globular or nearly globular structures which was clearly visible under a stereo microscope. The efficiency of PGRs and their concentrations were analyzed on the basis of visual observations (callusing percentage, callus growth etc.). Ineffective treatments were discontinued.

Proliferation of somatic embryos

For embryo proliferation, 40-50 mg callus was transferred on MS supplemented with different concentrations of BAP (1.0, 2.0, 4.0 mg/l) and 2,4-D (0.25, 0.50, 1.0, 2.0 mg/l). The embryogenic sectors were well maintained and proliferated by routine subculturing at 3-4 weeks intervals on the same medium. The culture showing somatic embryogenesis (embryogenesis percentage) and somatic embryo (number/ callus mass) were recorded.

Maturation of embryos

Embryogenic tissues with developing embryos were used for maturation of embryos. Maturation medium contained all the essential components of MS including various salts, vitamins. In some of the experiments, the medium was separately added with 0.5-1.0 mg/l abscisic acid (ABA) and 0.5-1.0 mg/l Gibberellic acid (GA_3). In GA_3 amended medium, somatic embryos became green and elongated (matured embryo) which germinated into plantlets later. The length of embryos was recorded after 4 and 8 weeks of culture.

Germination of embryos and plantlet formation

Mature somatic embryos were isolated after 8 weeks of culture and transferred to germination medium. This medium was of the same composition as that of initiation and maintenance medium, except the PGR part. The germination medium contained various concentrations of BAP (0.25, 0.50, 0.75, 1.0, 1.5, 2.0 mg/l). The numbers of somatic embryos germinated and converted into plants (root and epicotyl development) were recorded.

Various carbohydrates were also added to monitor the influence of sugar sources on embryo during conversion.

Regenerated somaclones were transferred into 250 ml conical flask containing about 100 ml of fresh nutrient medium (½ MS), 3% sucrose but without any PGR, and kept under the same cultural conditions as mentioned earlier. Regenerated plantlets were finally transplanted into small plastic pots, filled with soilrite, kept for another one week before transfer to the field.

Scanning electron microscopy (SEM)

The morphogenic development of somatic embryos was examined by Scanning Electron Microscopy (SEM). For SEM, embryogenic callus with developing embryos was fixed in 2% glutaraldehyde, adjusted to pH 6.8 in 0.1 M phosphate buffer for 24 h at 4ºC. The tissue was washed in the buffer, post-fixed for 2 h in similarly buffered 1% osmium tetroxide, dehydrated in a graded ethanol series and finally coated with gold palladium. The prepared samples were examined and photographed in a LEO 435 VP (Zeiss, Oberkochen, Germany) scanning electron microscope operating at 15-25 kV.

Statistical Analysis

All the data are expressed as mean ± standard error. Each of the experiment was repeated at least twice with three replicates. The results were determined using ANOVA followed by Least Significant Difference (LSD) test at $p<0.05$.

Results

Induction of embryogenic callus

On induction medium, callus initiation started to appear after 8 days of inoculation which was yellow (Fig. 1a), moderately compact and fast growing. All the tested combinations involving BAP, NAA and 2,4-D induced callus at varying percentages. The intensity of callus induction was maximum (84.71 ± 5.15) in medium containing 2.0 mg/l BAP + 0.25 mg/l 2,4-D + 1.0 mg/l NAA (Table 1) while embryogenic callus was found to be the least (25.32 ± 3.41) on medium containing higher concentrations of BAP (2.0 mg/l) with low 2,4-D level (0.50 mg/l). Proembryogenic Masses (PEM) or embryogenic calli were identified within 2-3 weeks of culture which proliferated fast and

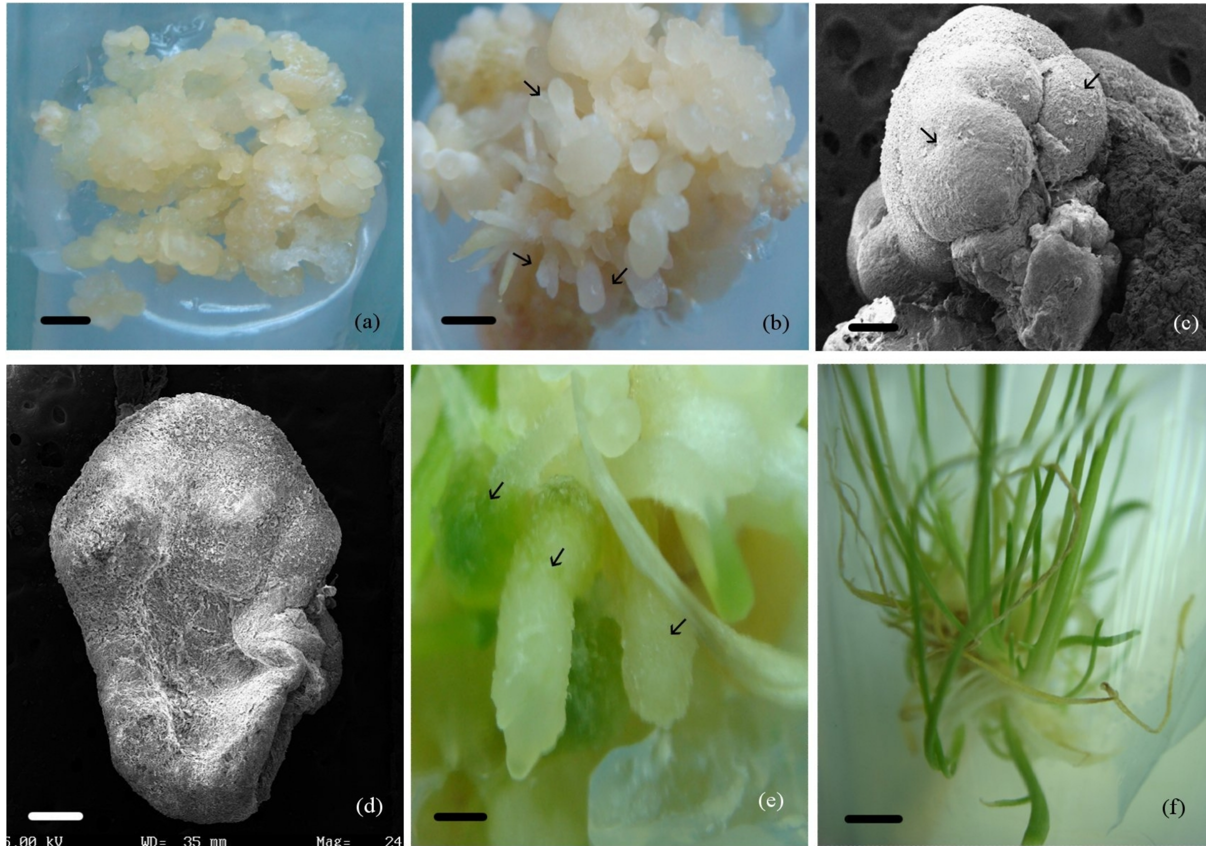

Figura 1. Inducción de callo, embriogénesis somáticay regeneración de planta en *Allium sativum* L. cv. Yamuna Safed 3. **a:** Callo inducido a partir de la parte basal del diente. **b:** Callo embriogénico mostrando numerosos embriones somáticos (flechas) en el medio de proliferación. **c & d:** SEM de dos embriones somáticos en dos etapas diferentes (flechas). **e:** Cultivo mostrando los embriones somáticos maduros verdes (flechas). **f:** Plántulas regeneradas a partir de los embriones somáticos. Escala: a, b & e: 1mm; c & d: 10µm; f: 1cm.

Figure 1. Callus induction, somatic embryogenesis and plant regeneration in *Allium sativum* L. cv. Yamuna Safed 3. **a:** a)Callus induced from basal part of clove. **b:** Embryogenic callus showing numerous somatic embryos (arrows) on proliferation medium. **c & d:** SEM of two somatic embryos at two different stages (arrows). **e:** Culture showing mature green somatic embryos (arrows). **f:** Somatic embryo regenerated plantlets. Bars: a, b & e: 1mm; c & d: 10µm; f: 1cm.

showed a gummy, mucilaginous appearance. PEM was a heterogeneous cluster of embryos, attached to the callus mass. The embryogenic calli were routinely maintained on the same induction medium.

Somatic embryo development and proliferation

Embryogenic calli, which were separated from the primary cultures, were transferred onto fresh MS basal medium for embryo development. Numerous somatic embryos of varied shapes were developed on the surface of the embryogenic callus (Fig. 1b). Morphologically, the early stages of embryos appeared as shiny globular structure, clustered together or formed in a scattered fashion on callus surface. As it developed further the embryos were marked by the presence of a notch at the median. Scanning electron microscopic examination (Figs. 1c & d) also revealed the same morphological nature where embryo with median notch was clearly visible. Some of the induced embryos were 'nearly normal' morphology which had reduced hypocotyls with developed shooty end. The highest average numbers of somatic embryos (173.71 per 200 mg of callus) was achieved after 8 weeks of culture on medium containing a combination of 1.0 mg/l BAP and 0.25 mg/l 2,4-D (Table 2). The number was, however, decreased with increasing concentration of 2,4-D. The study also indicated that the reduction of 2,4-D level in culture medium helped to promote a higher number of somatic embryos during subculture (data not shown). Growth of embryogenic callus with globular embryos was however, declined with increased concentration of BAP (2.0 mg/l or above) and 2,4-D (1.0 mg/l or above). Embryos were pro-

MS + PGR (mg/l)			Explants showing callusing (%)
BAP	2, 4-D	NAA	
0.00	0.25	0.00	45.94 ± 4.10[d]
0.00	0.50	0.00	45.74 ± 5.34[d]
2.00	0.25	0.00	26.32 ± 4.41[e]
2.00	0.50	0.00	25.32 ± 3.41[e]
2.00	0.25	0.25	60.88 ± 3.18[c]
2.00	0.25	0.50	69.21 ± 4.12[b]
2.00	0.25	1.00	84.71 ± 5.15[a]
2.00	0.50	1.00	82.71 ± 6.13[a]
2.00	1.00	1.00	78.71 ± 5.12[ab]
2.00	0.00	1.00	76.81 ± 3.98[b]
2.00	0.00	2.00	75.31 ± 4.98[b]
2.00	0.00	3.00	69.52 ± 4.11[bc]
		ANOVA	
F	-	-	20.24
P	-	-	0.000***
LSD 5 %	-	-	0.51

*Values are expressed as mean ± standard error. Within column, values are followed by the superscript letters are not significantly different at p = 0.05 level according to LSD test. F-test significant at ***p<0.001*

Tabla 1. Influencia de varias combinaciones de BAP, 2,4-D y NAA en la inducción del callo. Los datos fueron recogidos después de 6 semanas de cultivo.

Table 1. Influence of various combinations of BAP, 2,4-D and NAA in inducing callus. Data were scored after 6 weeks of culture.

MS + PGR (mg/l)			% of callus showing embryos	No. embryos/ 200 mg callus mass
BAP	2,4-D	NAA		
1.00	0.25	0.50	23.02 ± 2.30[d]	11.88 ± 2.02[d]
1.00	0.25	1.00	21.81 ± 3.20[d]	11.21 ± 4.44[d]
1.00	0.25	0.00	83.71 ± 5.12[a]	173.71 ± 4.12[a]
1.00	0.50	0.00	69.32 ± 4.41[a]	159.32 ± 5.11[a]
1.00	1.00	0.00	29.81 ± 3.98[c]	59.81 ± 3.18[bc]
1.00	2.00	0.00	15.31 ± 3.48[cd]	15.31 ± 2.98[d]
2.00	0.25	0.00	51.11 ± 4.12[b]	81.11 ± 3.12[b]
2.00	0.50	0.00	39.52 ± 3.11[bc]	52.52 ± 4.21[c]
2.00	1.00	0.00	14.50 ± 2.87[cd]	19.50 ± 2.17[d]
2.00	2.00	0.00	2.00 ± 0.11[e]	02.00 ± 0.31[e]
4.00	0.25	0.00	0.14 ± 0.11[e]	00.54 ± 0.09[e]
			ANOVA	
F	-	-	66.94	354.55
P	-	-	0.000***	0.015
LSD 5 %	-	-	0.543	0.677

*Values are expressed as mean ± standard error. Within columns, values are followed by superscript letters are not significantly different at p = 0.05 level according to LSD test. F-test is significant at: ***p<0.001; *p<0.05.*

Tabla 2. Porcentaje de callos mostrando embriogénesis y número de embriones para tratamientos con diferentes PGRs Los datos fueron recogidos después de 8 semanas de cultivo.

Table 2. Percentage of callus showing embryogenesis and embryo number in various PGRs treatments. Data were scored after 8 weeks culture.

duced in clusters but were easily separable. Table 2 summarizes the efficiency of various used PGRs in differentiating somatic embryos.

Maturation and germination of somatic embryos

Somatic embryo development was promoted on maturation medium which was amended with GA_3 or ABA (Table 3), Incorporation of GA_3 into the medium improved embryo quality; in this medium, the embryos were large, pigmented (Fig. 1e), grew in sizes, which later germinated into emblings. The highest embryo maturation frequency was obtained on medium supplemented with 0.5 mg/l GA_3, the maturity increased with time and, at the end of 8 weeks, over 61% cultured embryo matured. Higher level was however, not that effective. The influence of ABA was noted to be ineffective, did not produce any such morphological development and induced brown necrotic spots instead; therefore the treatments with ABA for embryo maturation were discontinued.

The effect of various carbon sources and their concentrations in maturation medium was evaluated. It was observed from the present study (Table 4) that the somatic embryos grew in size in all sources of carbon and the growth difference was not that significant except 3% maltose. The 3% level of carbohydrate was found to be more effective compared to 6% in terms of embryo growth.

Germination of somatic embryos

Somatic embryos were separated and transferred to germination medium that contained basal MS salts, vitamins, 3% sucrose and different concentrations of BAP (Table 5). The epicotyl emergence started to appear within 1-2 weeks of transfer,

MS + PGR (mg/l)		% Embryos showing maturation 4 weeks	8 weeks
GA_3	0.50	50.96 ±1.76^{a}	61.66 ± 3.80^{a}
	1.00	30.05 ±1.32^{b}	39.06 ± 2.28^{b}
ABA	0.50	13.16 ± 1.61^{c}	15.24 ± 1.43^{c}
	1.00	10.47 ± 0.64c	12.02 ± 1.23^{c}
		ANOVA	
F		269.75	92.52
P		0.001*	0.013*
LSD 5 %		0.167	0.416

*Values are expressed as mean ± standard error. Within columns, values are followed by superscript letters are not significantly different at p = 0.05 level according to LSD test. F-test is significant at: *p<0.05.*

Tabla 3: Influencia de GA_3 y ABA en la maduración embrionaria.
Table 3: Influence of GA_3 and ABA on embryo maturation.

later radicle developed; in others, the process was somewhat reverse. In still another type, the epicotyl and the radicle developed that grew simultaneously. After 6 weeks of culture, the germination frequencies were observed to be about 8-30%. The embryos subsequently developed a shoot and root axis, and formed a complete plantlet within 4-6 weeks of culture. In the absence of BAP, the frequency of conversion was rather low (8.02%). The addition of BAP (0.25 mg/l) increased the conversion by up to 21.87%. The highest conversion rate of 30.76% was observed at 0.5 mg/l BAP. The conversion rate was also decreased with increasing concentration of BAP, the same higher level also promoted abnormality.

It was evident that the maximum conversion of somatic embryos (65.15%) was noted in medium containing 3% fructose (Fig. 2), followed by 3% maltose (64.1%). No plantlet conversion was observed with medium supplemented with glucose (3 and 6%). At 3% glucose only shoot formation (devoid of root) of 10.55 % was noticed. The medium individually supplemented with 3% sucrose, 3% maltose and 3% fructose were observed to produce a higher plantlet conversion rate than the medium added with sugars of 6% level.

In somatic embryo regenerated plantlets no apparent morphological differences were observed. The regenerated plantlets (Fig. 1f) were kept *in vitro* for about 8-12 weeks before acclimatization in plastic pots filled with soilrite. The growth of these somaclones is being evaluated.

Treatment	Initial length (mm)	after 4 weeks (mm)	after 4 weeks (mm)
Sucrose 3 % (C) #	4.91 ± 0.5	7.61 ± 0.41^{b}	9.50 ± 0.30^{b}
Sucrose 6 %	4.62 ± 0.18	6.71 ± 0.53^{bc}	8.62 ± 0.22^{bc}
Maltose 3 %	5.41 ± 0.23	9.73 ± 0.32^{a}	10.98 ± 0.54^{a}
Maltose 6 %	5.31 ± 0.71	7.77 ± 0.50^{b}	9.82 ± 0.71^{b}
Glucose 3 %	5.11 ± 0.32	7.62 ± 0.99^{b}	9.12 ± 0.69^{b}
Glucose 6 %	5.01 ± 0.40	6.81 ± 0.57^{bc}	8.12 ± 0.48^{bc}
Fructose 3 %	4.89 ± 0.38	7.84 ± 0.52^{b}	9.62 ± 0.57^{b}
Fructose 6 %	4.85 ± 0.56	5.99 ± 0.52^{c}	7.97 ± 0.31^{c}
	ANOVA		
F		3.699	3.74
P		0.000 ***	0.001*
LSD 5 %		0.598	0.57

*Values are mean ± standard error where 3% sucrose (#) was considered as control. Within each column, values followed by the same superscript letters are not significantly different at p = 0.05 level according to LSD test. F-test significant at: ***p<0.001; *p<0.05.*

Tabla 4: Crecimiento de embriones somáticos en medio de maduración (MS + 0.5 mg/l GA_3) a los que se les añadió diferentes carbohidratos.
Table 4: Somatic embryo growth in maturation media (MS + 0.5 mg/l GA_3) which were also added with different carbohydrates.

BAP	Embryo conversion (%)
0.00	08.02 ± 1.87^{b}
0.25	21.87 ± 1.44^{ab}
0.50	30.76 ± 2.11^{a}
0.75	25.67 ± 1.98^{a}
1.00	19.99 ± 1.21^{ab}
1.50	16.01 ± 1.09^{b}
2.00	12.22 ± 1.03^{b}
ANOVA	
F	24.259
P	0.000***
LSD 5 %	0.51

Data are expressed as mean ± standard error mean.
*Within column, values followed by the same superscript letter are not significantly different at p = 0.05 level according to LSD test. F-test significant at:***p<0.001*

Tabla 5: Germinación de embriones osmaticos en varias concentraciones de BAP. Los datos fueron recogidos después de 6 semanas de cultivo.
Table 5: Somatic embryo germination in various concentrations of BAP. Data were scored after 6 weeks of culture.

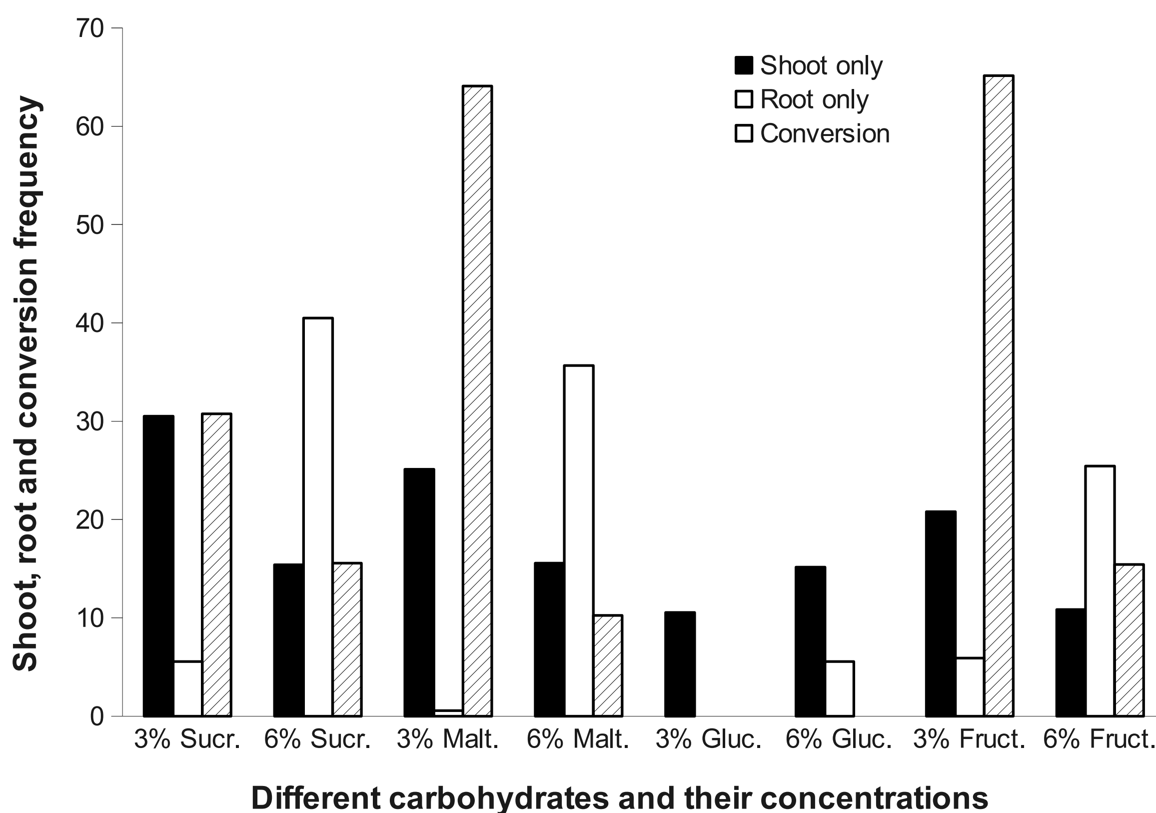

Figura 2. Influencia de los diferentes azúcares y sus concentraciones en la conversión a plántulas. Los embriones somáticos fueron cultivados en MS + 0.5 0.5 mg/l BAP. Los datos fueron recogidos después de 6 semanas de cultivo.

Figure 2. Influence of different sugars and their concentrations on plantlet conversion. Somatic embryos were cultured on MS + 0.5 mg/l BAP. Data were scored after 6 weeks of culture.

Discussion

In *Allium sativum* L. cv. Yamuna Safed 3, the embryogenic culture was initiated and the various stages of embryo development were studied in detail. Our results indicated that the rate of embryogenesis was quite high in *Allium* as was reported in many other plant groups (Klimaszewska et al. 2001, Pullman et al. 2003). The initiated cultures continued to proliferate for a period of two years or more making the culture a useful stable embryogenic source for future research purposes. Application of exogenous PGRs was found to be essential for the induction of callus, embryogenic culture establishment, proliferation, maturation and germination of embryos into plantlets.

Although calli production was noted to be maximum on medium amended with BAP + NAA + 2,4-D, individual application of 2,4-D was also very effective in inducing callus from basal clove explant. Generally, 2,4-D is considered to be one of the most important PGRs that regulate somatic embryogenesis *in vitro* (Zhang et al. 2007). During induction into the medium, 2,4-D increased explant's endogenous auxin level, one of the crucial signals that determine cultured cells' fate to become embryogenic (Victor 2005). Becwar et al. (1988) earlier reported that compared to higher concentrations, low levels of 2,4-D were more effective when combined with BAP for inducing embryogenic tissue. In auxin amended medium, cultured cell or tissue produce more ethylene than the auxin free cultures, which suppresses embryo development as the tissue multiplication continues to proceed without much check, the embryonic clumps develop into mature embryos only on medium amended with a very low level of 2,4-D (Razdan 1993). These observations support our present study that the auxin (2,4-D) has no significant effect on induction of embryos rather it has a considerable positive effect on callus production. Similar observation was noted in other plants like Melia where embryos were formed from pre-embryogenic determined cells, and did not depend on

2,4-D requirement (Evans et al. 1981, Litz & Schaffer 1987). In this present study, the application of BAP and NAA at high concentration (1.0-2.0 mg/l) with a low level of 2,4-D (0.25 mg/l) showed maximum influence in producing embryogenic callus.

Embryo maturation and simultaneous germination to obtain plantlets is one of the important steps in *in vitro* embryogenesis, which has been partially depending on embryo quality. Recently, a number of compounds like abscisic acid, sugar, sugar-alcohol, polyethylene glycol, activated charcoal, low temperature etc. have been added during embryo maturation and germination time (Lipavska & Konradova 2004, Robichaud et al. 2004). In our preliminary experiment, the presence of ABA was appeared to be ineffective for embryo maturation and germination as it prevented embryo maturation and reduced plant regeneration. This result is consistent with hybrid chestnut where addition of ABA was found to have no effect on embryo maturation (Vieitez 1995). In contrast, ABA-induced improved embryo maturation with quality embryo was reported in several studies of somatic embryogenesis particularly in conifers (Lelu-Walter et al. 1999, Maruyama et al. 2007). In this present cultivar of *Allium*, we observed that the introduction of GA_3 had significant influence on embryo maturation and germination. On GA_3 (0.5 mg/l) added medium somatic embryos grew in size and synthesized photosynthetic pigment, an early indicator of being autotrophic identity. These pigments may help to improve photosynthetic ability, extra storage reserves like lipid, triglycerides, protein and other important carbohydrates which favour fast *in vitro* germination.

The germination of somatic embryos has been determined by the quality of embryos. The morphology and biochemical reserves which are very similar to zygotic embryos generally produce normal plantlets. In our studied material, some of the induced embryos had 'nearly normal' morphology with stunted hypocotyls and developed shooty end, but these types of embryos showed poor rate of embryo germination. The quality of embryos, was however, improved on addition of various carbohydrates, like sucrose, maltose, glucose and fructose. In this variety of *Allium sativum*, the maturation and germination of somatic embryos were repressed by 3-6% glucose, 6% each of sucrose, maltose, and fructose; but application of fructose, maltose and sucrose at 3% individually were very effective. Similar observations were also observed in other plants where rapid somatic embryo maturation with 3% sucrose or maltose was noted (Corredoria et al. 2003). In several woody species, addition of maltose in culture induced fast embryo maturation (Druart 1990, Alemanno et al. 1997, Li et al. 1998). Although the relationship of maltose-enriched medium with early embryo maturation has not been established yet, it somehow caused low hydrolysis (Norgaard 1997) or it may induced a nutritional stress, which eventually improved embryo germination (Blanc et al. 1999). In *Catharanthus* G. Don (Apocynaceae), 3% fructose also showed a positive influence on maturation and germination of the cultured somatic embryos (Junaid et al. 2006). A similar mechanism might have played a role during maturation and subsequent germination of somatic embryos in the case of *A. sativum* L. It seems therefore that, in the present investigated variety, PGRs and carbon sources (3% each of fructose, maltose and sucrose) influenced embryogenesis by improving embryo size through cellular expansion and by extra accumulation of storage products as was earlier reported during embryo maturation (Merkle 1995). Variation in embryo size (length) could therefore, be considered a good marker for monitoring embryo maturation process. Maturation treatments that produced larger embryos not always yielded maximum plant recovery. The high and consistent embryogenic ability, may however, suggest a stable embryogenic cell lines, which could effectively be used in transgenic programme. Similarly, the regeneration methods established here may be used for fast *in vitro* propagation and cryopreservation purposes.

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