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Effects of explants and growth regulators in garlic callus formation and plant regeneration

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Abstract We intended to evaluate the effects of different explants and growth regulators on callus induction and plant regeneration in garlic (*Allium sativum* L.). Furthermore, we intended to differentiate among different morphological types of callus by light microscopy and to relate them with their abilities to regenerate plants in the red-garlic cultivar 069. A factorial design with BDS—basal Dunstan and Short (1977)—medium, as a control and supplemented with 0.042, 0.42 and 4.24 μM picloram or with 0.045, 0.45 and 4.5 μM 2,4-D, in both cases with and without 4.43 μM N^6-benzylaminopurine (BAP), was used. The cultures were grown in darkness at 25 ± 2°C and they were subcultured over a 6-month period. Basal plates and meristems were highly responsive explants, while immature umbels and root-tips were less responsive ones, as indicated by percentage of induced callus, growing callus and regenerating callus. The best response was 41% regenerating callus with 0.045 μM 2,4-D and BAP from basal plates while 57, 56 and 20% regenerating callus were obtained with 0.45 μM 2,4-D from meristems, root-tips and immature umbels, respectively. Also, these treatments showed a higher percentage of nodular and embryogenic callus (type I). Thus, it can be concluded that the use of meristems and 2,4-D will enhance callus production and quality, increase plant regeneration and allows to develop a protocol suitable for further transformation experiments in garlic.

Keywords *Allium sativum* · Benzyladenine · Picloram · 2,4-D · Histology · Somatic embryogenesis

Abbreviations

BAP	N^6-benzylaminopurine
BDS	Dunstan and Short medium (1977)
NAA	α-naphthaleneacetic acid
picloram	4-Amino-3,5,6-trichloropicolinic acid

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Genetic engineering and tissue culture protocols are efficient tools for complementing traditional garlic breeding programs and producing cultivars with higher yields, higher tolerance/resistance to viral and fungal diseases and better adapted to local environmental conditions (Barandiaran et al., 1999a, b, c; Martín-Urdíroz et al. 2004). Since Abo El-Nil (1977), somatic embryogenesis

and organogenesis were reported in garlic cultures (Myers and Simon 1998, 1999; Barandiarian et al. 1997, 1999a, b, c; Robledo-Paz et al. 2000; Luciani et al. 2001; Sata et al. 2000; Fereol et al. 2002). According to these reports, the most important factors affecting plant regeneration are the explant type, the physiological condition of the explant, the genotype and the growth regulator combination used in the culture medium. In this sense, for *Allium* species with asexual seed formation like garlic, immature umbels, bulblets and root fragments or root-tips are used with the objective of preserving parental genotypes for further use in breeding programs (Phillips and Hubstenberger 1987; Shahin and Kaneko 1986; Haque et al. 1997; Myers and Simon 1998; Barandian et al., 1999a, b, c; Robledo-Paz et al. 2000). Besides, callus differentiation and plant development are determined by growth regulators. In this respect, several reports showed the effects of picloram and 2,4-D in different garlic cultivars (Myers and Simon 1999; Barandarian et al., 1999b; Robledo-Paz et al. 2000; Sata et al. 2000; Fereol et al. 2002). In addition, histological studies in leek, onion and garlic described different callus types associated with plant regeneration and these observations were useful to select those calluses suitable for further subculture (Buitveld and Creemers-Molenaar 1994; Eady et al. 1998; Fereol et al. 2002). Therefore, we intended to evaluate the effects of different explants and growth regulators in a two-step protocol including callus induction and plant regeneration in garlic (*Allium sativum* L.). Furthermore, we intended to differentiate among different morphological types of callus by light microscopy and to relate them with their abilities to regenerate plants in the red-garlic cultivar 069.

Meristems, basal plates, immature umbels and root-tips from the red-garlic cultivar 069 (*Allium sativum* L.) were used as initial explants. A factorial design using BDS basal medium (Dunstan and Short 1977) as a control and supplemented with 0.042, 0.42 and 4.24 μM picloram or with 0.045, 0.45 and 4.5 μM 2,4-D in both cases with and without 4.43 μM BAP was used. Thirty explants were established in each treatment. The cultures were grown in darkness at 25 ± 2°C and subcultured monthly over a 6-month period. Callus induction was evaluated after 2 and 3 months. Callus growth was evaluated by the percentage of growing callus and their relative growth in the subsequent two subcultures. Plant regeneration was evaluated as the number of callus with shoots with respect to the total number of callus from basal plates and meristems after 6 months. To observe plant regeneration, calluses were subcultured in BDS medium with 4.43 μM BAP under a 16 h photoperiod with fluorescent light of ca. 50 μmol m^{-2} s^{-1}. Histological studies of the different morphological types of callus were also performed. Calluses were fixed in FAA solution (formol–acetic acid–ethanol), embedded in Paraplast®Plus (Sherwood Medical Co.), cut with a rotatory microtome in 10 μm sections and stained with safranine-fast green (Ruzin 1999). Statistical analyses of the percentage of induced callus, growing callus, and regenerating callus, were performed using a pairwise test comparing all treatments using a large-sample test of proportions (McClave and Sincich 2000). Besides, growth regulator and callus relative growth data were transformed and analyzed using the least square means for media comparisons (SAS Institute 1982).

According to the percentages of induced callus, growing callus and regenerating callus, different explants showed different responses. Thus, the best results were obtained with a continuous culture in a BDS medium with 0.045 and 0.45 μM 2,4-D with 4.43 μM BAP for meristems and basal plates after 5 months (Tables 1 and 2, respectively). The best results were also obtained in BDS medium with 0.45 μM 2,4-D without BAP for root-tips and immature umbels after 6 months. In these cases, only this treatment produced 20 and 57% regenerating callus (data not shown). Thus, root-tips and immature umbels were less responsive and showed delayed callus induction and plant regeneration compared with basal plates and meristems which performed better as initial explants. Therefore, the approach of using alternative explants for preserving parental genotypes proposed by Myers and Simon (1998) does not seem suitable for the red-garlic cultivar 069.

In line with the reports of Haque et al. (1997) and Zheng et al. (1998), we observed that 2,4-D

Table 1 Callus induction and callus percentage of growing callus, relative growth and regenerating callus from meristems in the red-garlic cv. 069 (*A. sativum* L.)

Meristems						
			Callus induction (%) 3 months	Callus percentage (%) 5 months		
				Growing callus	Relative growth	Regenerating callus
		μM				
BAP	Control		11.1^{bcd*}	65.2^{b}	167.6^{bcdef}	0.0^{cdef}
0μM	Picloram	0.042	7.1^{bcd}	63.6^{b}	163.4^{bcdef}	13.8^{bcdef}
		0.42	20.7^{bcd}	96.4^{ab}	167.1^{bcdef}	26.7^{abcde}
		4.24	96.3^{a}	96.7^{ab}	398.3^{bcd}	43.3^{abcde}
	2,4-D	0.045	0.0^{cd}	*	*	*
		0.45	100.0^{a}	100.0^{ab}	153.2^{bcdef}	55.6^{abc}
		4.5	100.0^{a}	100.0^{ab}	864.8^{a}	26.9^{abcde}
4.43 μM	Picloram	0.042	34.5^{bc}	100.0^{ab}	198.5^{bcdef}	0.0^{cdef}
		0.42	100.0^{a}	96.6^{ab}	136.9^{bcdef}	55.2^{abc}
		4.24	95.8^{a}	100.0^{ab}	76.1^{cdef}	15.0^{abcdef}
	2,4-D	0.045	46.7^{bc}	89.3^{ab}	*	*
		0.45	100.0^{a}	88.9^{ab}	124.7^{bcdef}	47.4^{abcd}
		4.5	100.0^{a}	100.0^{ab}	254.7^{bcde}	53.3^{abc}

*Different letters indicate significant differences at $P \leq 0.05$

Table 2 Callus induction and callus percentage of growing callus, relative growth and regenerating callus from basal plates in the red-garlic cv. 069 (*A. sativum* L.)

Basal plates						
			Callus induction (%) 3 months	Callus percentage (%) 5 months		
				Growing callus	Relative growth	Regenerating callus
		μM				
BAP	Control		33.3^{cdefgh*}	75.9^{abc}	62.7^{a}	13.6^{ab}
0μM	Picloram	0.042	3.7^{fgh}	57.7^{bc}	54.0^{a}	33.3^{ab}
		0.42	23.1^{efgh}	78.3^{abc}	48.3^{a}	5.6^{ab}
		4.24	82.6^{abcde}	91.3^{abc}	143.0^{a}	19.0^{ab}
	2,4-D	0.045	7.7^{fgh}	79.2^{abc}	47.1^{a}	31.6^{ab}
		0.45	70.8^{abcdef}	84.0^{abc}	154.6^{a}	0.0^{b}
		4.5	100.0^{abc}	93.3^{abc}	61.2^{a}	0.0^{b}
4.43μM	Picloram	0.042	53.3^{bcdefg}	89.7^{abc}	46.3^{a}	11.5^{ab}
		0.42	63.3^{bcdefg}	58.3^{abc}	28.5^{a}	0.0^{b}
		4.24	69.2^{bcdef}	79.2^{abc}	26.7^{a}	0.0^{b}
	2,4-D	0.045	55.0^{bcdefg}	100.0^{ab}	22.0^{a}	40.9^{a}
		0.45	50.0^{bcdefg}	100.0^{ab}	293.2^{a}	20.8^{ab}
		4.5	100.0^{abc}	83.3^{abc}	106.6^{a}	0.0^{b}

*Different letters indicate significant differences at $P \leq 0.05$

was more effective than picloram in callus induction and plant regeneration regardless of explant type. These results confirmed previous results obtained from a preliminary experiment with cultivars Español Selección Ascasubi and I50, where the best callus induction was obtained in BDS medium with 0.45 μM 2,4-D and 4.43 μM BAP (data not shown). Thus, our protocol was clearly optimized for using meristems as initial explants in a BDS medium with 0.45 μM 2,4-D and 4.43 μM BAP and for producing 100% induced calluses and 47% regenerating calluses in

6 months. These results are similar to those obtained by Barandiarian et al. (1999a), who produced 72% induced calluses and less than 30% regenerating calluses for the red-garlic group in 7 months.

Besides, we characterized three different morphological types of callus by direct and histological observations. They were called callus type I, II and mixed (Fig. 1). These three callus types were similar to those calluses obtained in leek by Buitveld et al. (1993) and in onion by Eady et al. (1998). Moreover, our observations about callus type I are consistent with the garlic embryogenic callus described by Fereol et al. (2002) and their developmental pattern was similar to the pattern found by Eady et al. (1998). Specifically, a first step with callus type I holding early embryos in the periphery (Fig. 1.I) was followed by two later steps where the callus became older, oxidized and lost its initial embryogenic ability (i.e. mixed and type II) (Fig. 1.II and III, respectively). Furthermore, high auxin concentrations produced a decrease in the percentage of callus type I favoring a higher percentage of callus mixed or type II irrespective of BAP addition and explant type (data not shown). Thus, both regeneration pathways, i.e. shoot and embryo regeneration, were observed at different times in the same

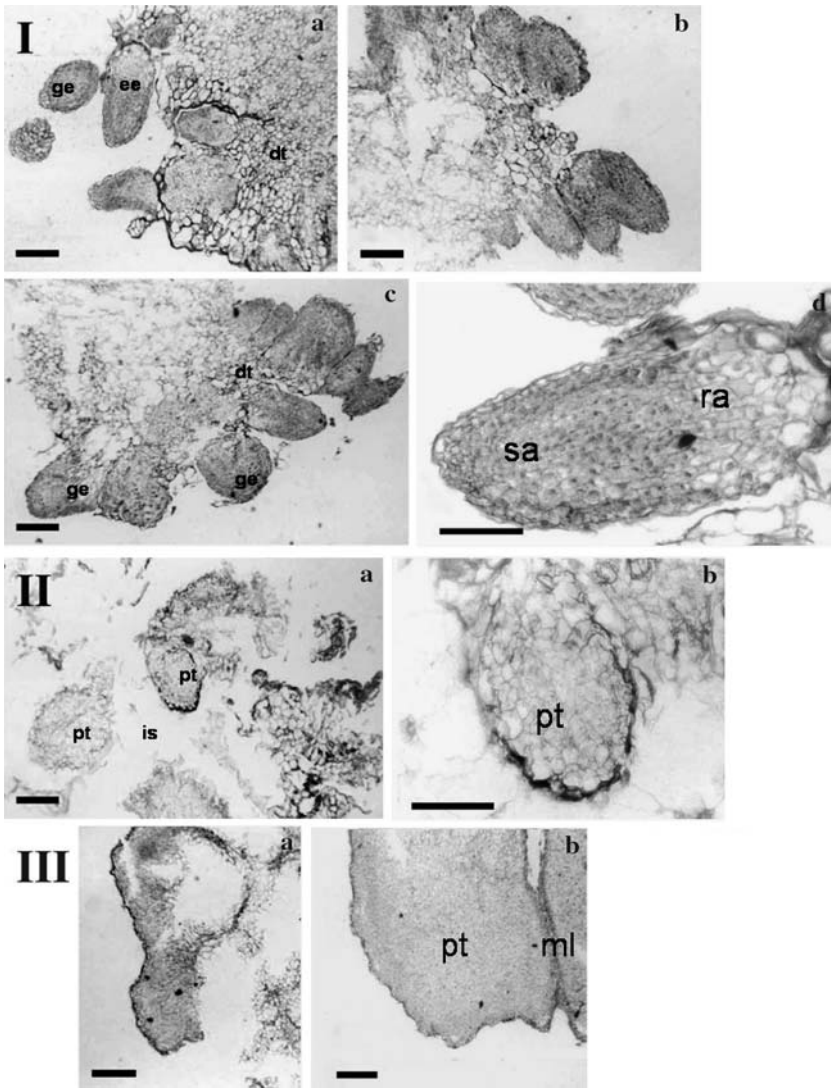

Fig. 1 Different morphological types of callus obtained from basal plates and meristems in the red-garlic cv. 069 (*A. sativum* L.): (**I**) type I: nodular and embryogenic callus (**a**–**c**), *ge* globular embryos, *ee* elongated embryos, *dt* differentiated tissue, (**d**) elongated embryo, *sa* future shoot apex, *ra* future root apex; (**II**) type II: dispersed and watery callus (**a**,**b**) *ic* intercellular space, *pt* parenchymatic tissue, and (**III**) type mixed: transition callus with parenchymatic tissue and superficial meristematic layers (**a**,**b**) *ml* meristematic layers

callus. Moreover, embryo induction occurred at early stages while shoot regeneration occurred at later stages. In this sense, De Klerk et al. (1997) and Guohua (1998) reported that auxins induce callus formation and proliferation and somatic embryogenesis while cytokinins induce mostly shoot and root differentiation and elongation. Therefore, we conclude that the use of meristems as initial explants and 2,4-D as the appropriate growth regulator will enhance callus induction and growth producing friable callus with the capacity of regenerating plants either by embryo or shoot formation.

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