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EFFICIENT PLANT REGENERATION OF GARLIC (*ALLIUM SATIVUM* L.) BY ROOT-TIP CULTURE

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Summary

Root apices from *in vitro* cultured garlic (*Allium sativum*) cloves of cvs. ABEN and GT96-1 were used as axenic explants for organogenic callus production and plant regeneration experiments. Explants cultured in media based on those of Chu and co-workers (N6) or Murashige and Skoog (MS) could induce organogenic callus after 8 wk culture in darkness. Both media were supplemented with 2,4-dichlorophenoxyacetic acid (2.2–4.5 μ*M*), alone or combined with 6-furfurylaminopurine (kinetin, 2.3–4.6 μ*M*). Shoots started to grow 3 wk after culturing in the presence of light and the addition to culture media of 4.4 μ*M* N^6-benzyladenine. Plants capable of producing microbulbs regenerated 6 wk later. Up to 170 plants g^{-1} FW callus were obtained when culturing was initiated in MS medium supplemented with 4.6 μ*M* kinetin and 4.5 μ*M* 2,4-dichlorophenoxyacetic acid.

Key words: bulb induction; callus induction; organogenesis; tissue culture.

Introduction

Garlic (*Allium sativum* L., Liliaceae, subfamily Allioideae) is a monocotyledonous crop used worldwide as a condiment and for its medicinal properties. In most growing areas it is susceptible to diseases caused by viruses, nematodes and fungi, and suffers from insect pests. In Mexico white rot, caused by the fungus *Sclerotium cepivorum*, is particularly widespread, affecting production considerably and sometimes destroying complete garlic fields (Laborde, 1987; Davies, 1994). Garlic cultivars can only be propagated vegetatively, and their multiplication rates are fairly low. This mode of reproduction facilitates disease transfer (Novák, 1990) and limits breeding efforts to the slow screening and selection of spontaneous or induced mutations. A very promising complementary breeding approach is the production of transgenic garlic plants that are resistant to one or more diseases of importance. To date, there have been some reports on the *in vitro* regeneration of garlic plants by organogenesis (Havránek and Novák, 1973; Kehr and Schaeffer, 1976; Bhojwani et al., 1982; Nagakubo et al., 1993; Ma et al., 1994; Mohamed-Yassen et al., 1994; Haque et al., 1997; Ayabe and Sumi, 1998; Myers and Simon, 1998), or embryogenesis (Abo El-Nil, 1977; Myers and Simon, 1998; Al-Zahim et al., 1999). However, there is as yet no consistent, reliable regeneration system that could be used effectively for the genetic transformation of garlic. We report the development of an efficient garlic plant regeneration system by root-tip culture.

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Materials and Methods

Plant material. This was obtained from mature cloves of the commercial cvs. ABEN and GT96-1, grown in El Bajío State of Guanajuato, Mexico.

Culture media. Medium A was prepared with half the concentration of the salts and vitamins found in that of Murashige and Skoog (1962) (MS), with 29.2 m*M* sucrose and 7 g l^{-1} Phytagar (Gibco BRL, Life Technologies Rockville, MD, USA). Media B and C were prepared with salts from Chu et al. (1975) (N6); vitamins from Eriksson (1965); 58.4 m*M* sucrose; 6 m*M* L-proline; and 2.2 μ*M* (in B) or 4.5 μ*M* (in C) of 2,4-dichlorophenoxyacetic acid (2,4-D). Medium D contained the salts and vitamins of MS, 87.6 mM sucrose, 4.5 μ*M* of 2,4-D and 4.6 μ*M* of 6-furfurylamino purine (kinetin). Media E and F contained the salts and vitamins of MS; 87.6 m*M* sucrose; 2.2 μ*M* 2,4-D; and 2.3 μ*M* kinetin (E) or 3.3 μ*M* 2,4-D (F). Medium G (development) contained the salts and vitamins of MS; 58.4 m*M* sucrose; and 4.4 or 2.2 μ*M* N^6-benzyladenine (BA). Medium H was identical to G, except for the exclusion of BA.

For all media the pH was adjusted to 5.8, and they were sterilized in an autoclave at 1.1 kg cm^{-2}, 121°C, for 20 min.

Induction of organogenic callus. Garlic cloves were separated from the compound bulb and pealed manually before treatment in a solution of Ridomil Bravo, 3.0 g l^{-1} (9.0% metalaxyl, 72.0% chloratonyl); Benlate 1.2 g l^{-1} (50% benomyl in solution); and Agrimycin 100, 1.0 g l^{-1} (18.7% streptomycin, 2.0% oxytetracyclin). Cloves were then transferred to a chlorinated solution (Cloralex, 1.8% active chlorine) for 20 min and rinsed three times in sterilized distilled water. Disinfected cloves were laid on sprouting medium (A), and after 3 d, 3-mm-long root-tip segments were cut off with a scalpel. The latter were cultured for 8 wk at 25 ± 2°C in complete darkness, in 2.5 × 10 cm glass tubes containing 10 ml of different media (B, C, D, E or F) for inducing organogenic callus (30 tubes per treatment).

Shoot growth. After 8 wk culture on shoot-induction medium, calluses were transferred to 250 ml glass jars containing 30 ml medium G with 4.4 μ*M* BA, under a 16 h photoperiod (50 μmol m^{-2} s^{-1} irradiance). All calluses were subcultured twice a fortnight in the same medium, and those having small regenerated shoots were finally subcultured onto medium H to induce plant development.

Regeneration efficiency of organogenic callus. About 500 mg of callus obtained from each of the five induction treatments were placed in 250 ml glass jars containing 30 ml medium G supplemented with 4.4 μ*M* BA, for

30 d under a 16 h photoperiod. They were then subcultured for 15 d, under similar conditions, in medium G supplemented with 2.2 μ*M* BA, and were finally transferred to medium H for estimation of the proportion of shoots per g callus FW.

Statistical analysis. The experimental set-up was completely randomized with two replications for analysis of the number of explants with callus, and the number of explants with shoots. Eleven replications were obtained for number of shoots per g callus. ANOVA and Tukey's Studentized range were used. Data were analyzed using the generalized linear models procedure of the Statistical Analysis System (SAS).

Results and Discussion

The induction of callus derived from root-tip explants and the differentiation of adventitious shoots was achieved in the five media assayed and in both cultivars tested. Statistically there were no significant differences between media and genotype for the number of explants that induced callus or the number of calluses that formed adventitious shoots. Nevertheless, the frequency of callus induction varied from 63 to 100%, and the differentiation of shoots was from 20 to 100%; the higher average value was obtained with the combination of cv. ABEN and the N6 medium plus 2.2 μ*M* 2,4-D (medium B). Therefore the number of explants that formed callus, and the number of calluses that differentiated into shoots, were independent of the cultivar and medium employed. Concerning the number of regenerated shoots per g FW, statistically there were highly significant differences for the combination, cultivar and culture medium. Table 1 shows Tukey's test values: the best treatment was the combination of cv. ABEN and the medium with 4.5 μ*M* of 2,4-D plus 4.6 μ*M* kinetin (medium D), in which it was possible to obtain nearly 170 shoots per g callus FW. At the same statistical level is the combination of cv. ABEN with MS medium plus 2.2 μ*M* 2,4-D and 2.3 μ*M* kinetin (medium E). The numerical differences were considerable because, with the combination of MS medium plus 2.2 μ*M* 2,4-D and 2.3 μ*M* kinetin, only 80 shoots per g callus FW were obtained, in contrast to 170 shoots obtained in medium D. Our results indicate that the number of regenerated shoots per g FW callus were according to the combination of plant growth regulators supplementing the culture media used for the induction of callus. Cytokinins appear to have a specific effect during the regeneration phase in the protocol presented here. This is in agreement with that reported by Haque et al. (1997), in which only auxins in combination with cytokinins induced shoot regeneration. In fact, in any callus cultured in medium devoid of growth hormones, or supplemented only with auxins, shoots never developed into plantlets.

Organogenic callus was induced in culture media D, E or F, which were based on MS medium supplemented with different concentrations of kinetin and 2,4-D, as well as in media B and C, which were based on medium N6 supplemented with 2,4-D and L-proline, as has been reported for maize scutellar explants (Armstrong and Green, 1985). The parallel response between these two different monocot species may indicate a simple coincidence or, more interestingly, that the pertinent culture media included a mixture of nutrients and growth regulators that might prove successful for a range of other monocot species, using different tissue explants.

The callus induction process began when root-tip segments became swollen after 15 d culture on inducing media. Later, cellular proliferation began and was limited to the meristematic zone of all root-tip explants, confirming the observations made by Haque et al. (1997) who found that neither shoots nor callus developed from the cut surface, non-meristematic part of the explant. This proliferation resulted in the formation of distinct callus by the end of the eighth week (Fig. 1A), which was small and either pale yellow or beige. Callus mass and size increased considerably on transfer to MS medium plus 4.4 μ*M* BA (medium G), in which they developed adventitious shoots after 3 wk. Shoots were easily discernible by weeks 5–6 of culture in medium G (Fig. 1B). Only those shoots that grew from parts of the callus nearest the culture medium achieved full development. These developed into regenerated plantlets, with both shoot and roots, in growth medium G (Fig. 1C, D). The small bulbs that began to form 10 wk later (26 wk from the start of the experiment; Fig. 1E), were transferred successfully to soil.

Callus induction and plant regeneration have been shown to be possible in a number of *Allium* species (Novák et al., 1986). Regeneration in *Allium* has mostly been attempted by organogenesis on species such as *A. cepa* (Dustan and Short, 1978); *A. fistolosum* (Shahin and Kaneko, 1986); *A. carinatum* (Havel and Novák, 1988); and *A. ampeloprasum* (Shavemaker and Jacobsen, 1995). In the case of garlic (*A. sativum*), shoot differentiation has been successfully induced in some cultivars (Kehr and Schaeffer,

TABLE 1

EFFECTS OF CULTURE MEDIA ON INDUCTION OF ORGANOGENIC CALLUS AND REGENERATION OF PLANTLETS FROM GARLIC ROOT-TIP EXPLANTS AFTER 17 WK CULTURE

Culture media	Cultivar	Explants with callus	Callus with shoots	No. shoots g^{-1} FW[a]
N6 + 2.2 μ*M* 2,4-D (B)	ABEN	27.5a	21.0a	11.4b
	GT96-1	21.5a	19.5a	nd
N6 + 4.5 μ*M* 2,4-D (C)	ABEN	19.0a	18.5a	nd
	GT96-1	20.0a	12.0a	41.3b
MS + 4.5 μ*M* 2.4-D; 4.6 μ*M* kinetin (D)	ABEN	30.0a	19.0a	169.8a
MS + 2.2 μ*M* 2,4-D; 2.3 μ*M* kinetin (E)	ABEN	30.0a	19.0a	79.9ab
	GT96-1	28.0a	9.0a	nd
MS + 3.3 μ*M* 2,4-D (F)	ABEN	30.0a	6.0a	nd
	GT96-1	nd	nd	40.1b
Tukey's Studentized range		33.3	28.9	101.4

[a]FW, fresh weight of callus; nd, not determined.

Means with the same letters are statistically equal (Tukey's test, $\alpha = 0.05$).

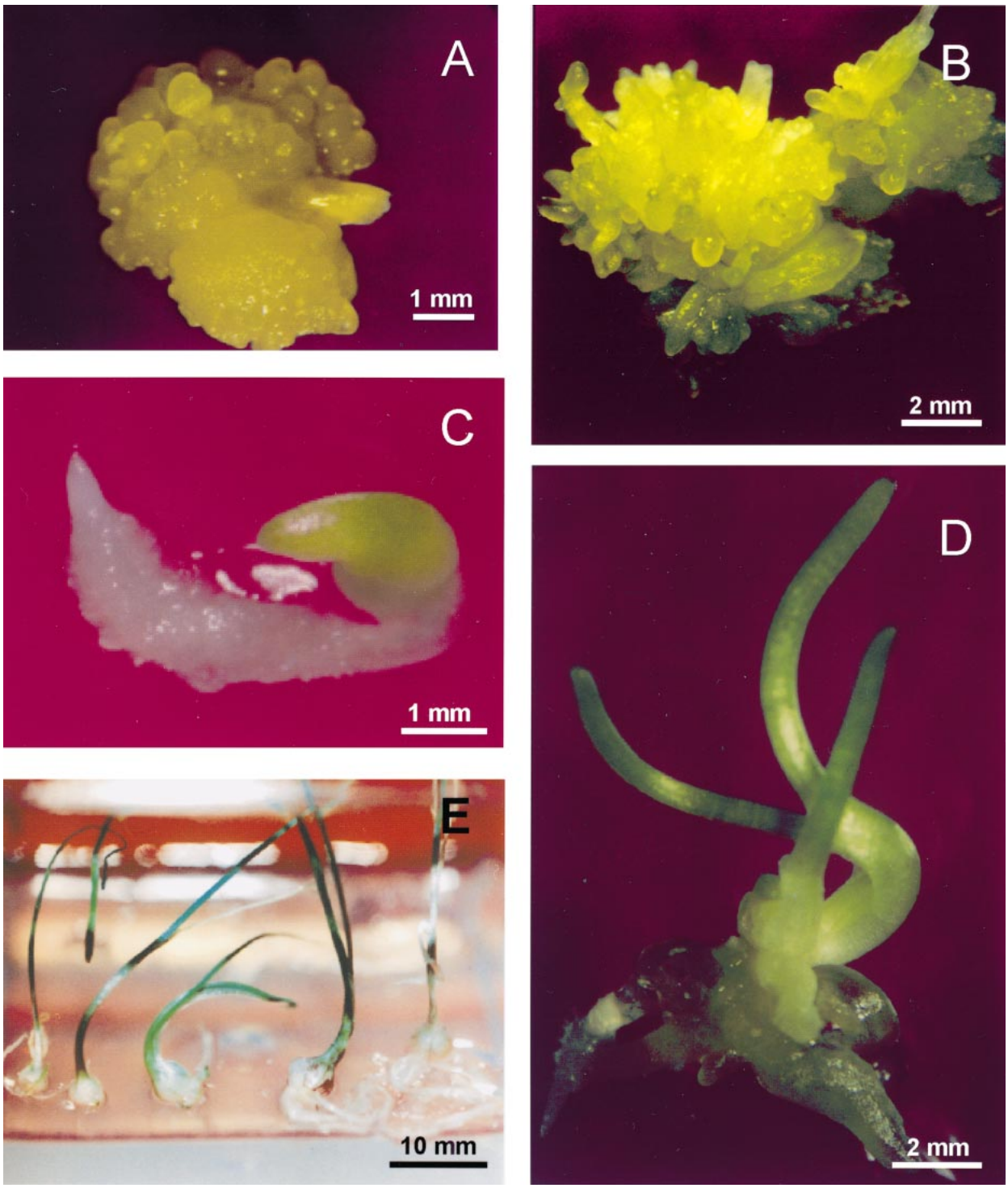

FIG. 1. Shoot differentiation and plantlet regeneration from garlic (*Allium sativum*) root-tip explants. (A) Organogenic callus after 8 wk culture in darkness on medium D. (B) Shoot differentiation after 5 wk culture on medium G. (C, D) Regenerated plants after 15 and 17 wk culture, respectively. (E) Fully regenerated plants with microbulbs.

1976; Abo El-Nil, 1977; Myers and Simon, 1998; Nagakubo et al., 1993; Ma et al., 1994; Barandiaran et al., 1999a,b), but the corresponding efficiency of plant regeneration has not been reproducibly high.

The protocol reported here for the regeneration of garlic plants by adventitious shoots has the following advantages over published methods. (i) It is possible to obtain up to 170 shoots g^{-1} callus FW by simply dividing and subculturing during the early stages of plant

regeneration; this regeneration rate is clearly higher than the best results (16 g^{-1} callus FW) reported to date (Barandiaran et al., 1999b). (ii) Using root tips as explant greatly increases the regeneration potential that can be achieved over other explant types such as shoot tips (Nagakubo et al., 1993; Mohamed-Yassen et al., 1994), from which it is possible to obtain only one explant per clove. In contrast, some varieties of garlic produce 30 or more root tips per clove, and it would be possible to regenerate more than 900 shoots per clove in a 6 mo. period. This efficiency is higher than Nagakubo et al. (1993) reported using shoot tips (137.7 shoots per explant in 27 wk). (iii) Plant regeneration can be achieved within a period of 4 mo. as opposed to the 7–9 mo. reported for root-tip explants obtained in a different way (Myers and Simon, 1998). (iv) Organogenic callus showed a high, sustained growth rate, as long as it was subcultured at regular intervals (15 d).

From the sum of these advantages, it can be suggested that the protocol presented in this paper should provide an appropriate source of material for the efficient genetic transformation of garlic, either by the particle bombardment method and/or the *Agrobacterium tumefaciens* system. The shorter process proposed here allows a higher throughput for a faster, larger-scale transformation and screening protocol.

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