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Regeneration of garlic callus as affected by clonal variation, plant growth regulators and culture conditions over time

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Abstract A long-term regeneration system for garlic (*Allium sativum* L.) clones of diverse origin was developed. Callus was initiated on a modified Gamborg's B-5 medium supplemented with 4.5 μM 2,4-D and maintained on the same basal medium with 4.7 μM picloram + 0.49 μM 2iP. Regeneration potential of callus after 5, 12 and 16 months on maintenance medium was measured using several plant growth regulator treatments. The 1.4 μM picloram + 13.3 μM BA treatment stimulated the highest rate of shoot production. Regeneration rate decreased as callus age increased, but healthy plantlets from callus cultures up to 16-months-old were produced for all clones. Regeneration of long-term garlic callus cultures could be useful for clonal propagation and transformation.

Key words *Allium sativum* · Benzyladenine · Picloram · Thidiazuron · 2,4-D · 2iP

Abbreviations *Picloram*: 4-Amino-3,5,6-trichloropicolinic acid · *BA*: N^6-benzyladenine · *NAA*: α-Naphthaleneacetic acid · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *2iP*: 6-(γ-γ-Dimethylallylamino) purine · *TDZ*: Thidiazuron

Introduction

Abo El-Nil (1977) was the first to report that regeneration in garlic involved both organogenesis and embryogenesis. The regeneration of garlic plants from callus cultures has been reported (Abo El-Nil 1977; Nagasawa and Finer 1988; Pandey et al. 1996), but these studies involved only regeneration from short-term callus cultures of one clone each. Kehr and Shaeffer (1976) reported the differentiation of garlic callus into whole plants using three clones but did not report on the frequency of regeneration for any of the clones. Barandiaran et al. (1999) recently evaluated regeneration in 20 garlic clones after 3 months of culture. Regeneration of protoplasts into whole plants from one clone through a callus stage was reported by Ayabe et al. (1995). Novak (1980) evaluated regeneration of long-term callus cultures using one clone and found that organogenic potential decreased and genetic instability increased greatly in cultures that were maintained on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) for longer than 120 days.

A garlic regeneration system needs to be applicable to a wide range of diverse clones to be broadly useful. Also, since garlic grows slowly in culture (Novak 1990), regeneration capability needs to be retained on long-term callus cultures for both transformation and clonal propagation. The study presented here investigated the use of several plant growth regulator treatments to promote the regeneration of garlic callus using clones of diverse origin grown in vitro for up to 16 months.

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Materials and methods

Plant material

The garlic (*Allium sativum* L.) clones 'M/V', 'PI383819', 'Piacenza', 'Creole Red' and 'RAL27' were used to test the effect of plant growth regulator treatments on the regeneration of callus. These clones are of diverse origin and isozyme patterns and have varying plant morphological characteristics when grown in Madison, Wisconsin (Pooler and Simon 1993).

Callus induction and maintenance

Garlic cloves were surface-sterilized by removing the outer protective leaf sheath, placing them in 95% ethanol for 5 min,

transferring them to a 10% commercial bleach solution plus Tween 20 (1 drop for every 50 ml) for 20 min, followed by four washes with sterile water. The cloves were transferred to a sterile paper towel, the storage leaf was removed and the first primordial leaf surrounding the shoot tip was excised, cut into four pieces and transferred to callus induction medium [modified Gamborg's B-5 basal medium (Gamborg et al. 1968) supplemented with KNO_3 (0.16 m*M*), $NH_4H_2PO_4$ (2.0 m*M*), NH_4NO_3 (1.9 m*M*), 3% sucrose, 2,4-D (4.5 μ*M*), pH 5.8, and solidified with 0.8% Difco bacto agar]. For each clone, ten cloves of similar size and mass from each of ten different bulbs were introduced to culture. Thus, 40 initial explants were initiated for each clone. Of these, 28 were maintained throughout these experiments and used randomly for each experiment. Distinct explant sources were recorded for each callus line. Callus was grown in the dark at 24 °C and subcultured every 4 weeks. After 8 weeks in culture on 2,4-D-containing medium, callus was transferred to the same basal medium except that the plant growth regulators 4-amino-3,5,6-trichloropicolinic acid (picloram) (4.7 μ*M*) and 6-(γ-γ-dimethylallylamino) purine (2iP) (0.49 μ*M*) were used instead of 2,4-D to improve callus proliferation. Callus production was maintained on medium supplemented with picloram plus 2iP using the growing conditions described above.

Regeneration overview and from 5-month-old callus

Regeneration was evaluated with 5-, 12-, and 16-month-old callus. There was special emphasis placed on screening a broad range of plant growth regulators at 5 months, determining Thidiazuron (TDZ) effects at 12 months and optimizing growth regulator concentration at 16 months.

Friable 5-month-old callus from the garlic clone 'Piacenza' maintained on 4.7 μ*M* picloram plus 0.49 μ*M* 2iP was used for regeneration. Four callus pieces (each about 0.5 cm^3) from different initial explants were each transferred to a 100 × 15 mm petri plate containing the same basal medium described above supplemented with ten combinations of plant growth regulators. A completely randomized design with subsampling was used with 16 replications, i.e. four petri plates per treatment . Treatments included: (1) control – no plant growth regulators; (2) α-naphthaleneacetic acid (NAA) (1.6 μ*M*); (3) picloram (1.4 μ*M*); (4) 2iP (14.8 μ*M*); (5) N^6-benzyladenine (BA) (13.3 μ*M*); (6) 2iP (14.8 μ*M*) + BA (13.3 μ*M*); (7) picloram (1.4 μ*M*) + 2iP (14.8 μ*M*); (8) picloram (1.4 μ*M*) + BA (13.3 μ*M*); (9) NAA (1.6 μ*M*) + 2iP (14.8 μ*M*); (10) NAA (1.6 μ*M*) + BA (13.3 μ*M*). The petri plates were randomly placed under cool-white fluorescent lights (95 μmol m^{-2} s^{-1}) with 16-h days and a temperature range of 24 °C to 28 °C (dark to light). Explants were subcultured every 4 weeks to fresh medium, and data were collected after 4 months. Shoot regeneration (percentage of explants with at least one shoot) and the number of shoots per explant (number of shoots on explants with at least one shoot) were measured. Root regeneration (%) was measured in a similar fashion, but the data are not presented in this report. Callus from each treatment which induced shoot regeneration was transferred to the basal medium without plant growth regulators, and from 5 to 15 whole plants for each were grown for field evaluation.

Regeneration from 12-month-old callus

Regeneration was evaluated on 12-month-old callus of 'Piacenza' as well as the clones 'M/V', 'PI383819', 'RAL27' and 'Creole Red'. The same basal medium as described above was used with these plant growth regulator treatments: (1) control – no plant growth regulators; (2) picloram (1.4 μ*M*) + BA (13.3 μ*M*); (3) picloram (3.5 μ*M*) + BA (8.9 μ*M*); (4)*N*-phenyl-*N*'-1,2,3-thidiazol-5-ylurea (Thidiazuron or TDZ) (0.01 μ*M*); (5) TDZ (0.025 μ*M*); (6) TDZ (0.04 μ*M*); (7) TDZ (0.075 μ*M*); (8) 6-furfurylaminopurine (kinetin) (4.7 μ*M*). The first two treatments were identical to treatments (1) and (8) in the 5-month regeneration of callus. TDZ was filter-sterilized and added to the medium after autoclaving. Petri plates were kept under the same growing conditions as described above with subculturing every 4 weeks. A randomized complete block design with 16 replications as above and four blocks was used, and data were collected after 4 months. From 5 to 15 plantlets from each clone and treatment which induced shoot regeneration were grown to complete plants for field evaluation as described for 5-month-old callus.

Regeneration from 16-month-old callus

Sixteen-month-old callus from the garlic clones 'PI383819', 'Piacenza', 'RAL27' and 'Creole Red' was used in this experiment to evaluate the concentration effect of picloram and BA. A randomized complete block design with 16 replications as above and nine blocks was used. The same basal medium and growing conditions were used as described above with the plant growth regulator combination of picloram plus BA at these concentrations: (1) control – no plant growth regulators; (2) picloram (1.4 μ*M*) + BA (13.3 μ*M*); (3) picloram (3.5 μ*M*) + BA (8.9 μ*M*); (4) picloram (0.47 μ*M*) + BA (11.1 μ*M*). The first three treatments were the same as in the 12-month regeneration of callus. Data were collected after 4 months. From 5 to 15 plantlets for each clone and treatment which induced shoot regeneration were grown to complete plants for field evaluation as described for 5-month-old callus. Five isozymes, esterase (EST, 3.1.1.1), glucose-phosphate dehydrogenase (G6PDH, 1.1.1.49), phosphoglucomutase (PGM, 5.4.2.2), 6-phosphogluconate dehydrogenase (6PGDH, 1.1.1.46) and shikimate dehydrogenase (SKDH, 1.1.1.25) were analyzed (Pooler and Simon 1993) in regenerated and bulb-propagated plants.

Statistical analysis

Data were analyzed using the general linear models (GLM) procedure of the SAS statistical package (SAS Institute 1982). For regeneration frequency, the data were subjected to square root and logarithmic transformation for analysis. Mean separation and standard error calculation were performed using least square means of SAS (SAS Institute 1982).

Results and discussion

All five garlic clones of diverse origin regenerated and produced healthy plantlets on medium supplemented with 1.4 μ*M* picloram + 13.3 μ*M* BA after 16 months of culture as callus. Shoot production varied significantly between clones ($F = 5.5$, $P = 0.0002$). 'PI383819' and 'RAL27' had better overall shoot production than 'M/V', 'Piacenza' or 'Creole Red'. Variation between explants within a clone was of a magnitude typical for *Allium* tissue culture (Myers and Simon 1998; Phillips and Luteyn 1983; van der Valk et al. 1992) and was not significant for shoot numbers per explant or shoot regeneration (%) in any of the experiments.

The use of 4.5 μ*M* 2,4-D has been reported to inhibit garlic regeneration and increase genetic instability with extended exposure (120–240 days; Novak 1980). In the current study, the exposure of the callus to 2,4-D for 8 weeks for callus initiation did not inhibit regeneration; all callus with shoot regeneration produced roots on growth regulator-free medium, and whole plants were able to be produced for field evaluation. Furthermore,

in 513 regenerated plantlets representing all five clones, no variation in isozymes profiles among plants for a given clone was observed for five enzymes, and no obvious morphological differences were observed upon visual comparison in the field to plants generated from bulbs of the same clones. Replicated field trials of regenerants over several years and further molecular marker testing will be necessary to confirm this observation.

Regeneration from 5-month-old callus

The 1.4 μM picloram + 13.3 μM BA treatment produced the highest shoot numbers and regeneration (%) with about 94% of the explants producing shoots with a mean of 3.8 shoots per explant for the clone 'Piacenza' (Fig. 1). Callus grown on medium with either auxins or cytokinins alone produced no shoots. The control, (2iP + BA), (NAA + 2iP) and (NAA + BA) treatments were comparable for the mean number of shoots per explant and shoot regeneration (%). (Picloram + 2iP) stimulated comparable shoot regeneration (%) to the aforementioned group.

It has been reported for onion that picloram is more effective than other auxins in promoting somatic embryogenesis, callus maintenance and regeneration for extended periods of time in culture (Phillips and Luteyn 1983). In the current study, we observed the same results in garlic, with picloram in combination with BA or 2iP producing more shoots per explant than either of the NAA combination treatments (Fig. 1). Treatments with picloram alone or BA alone did not promote regeneration of shoots.

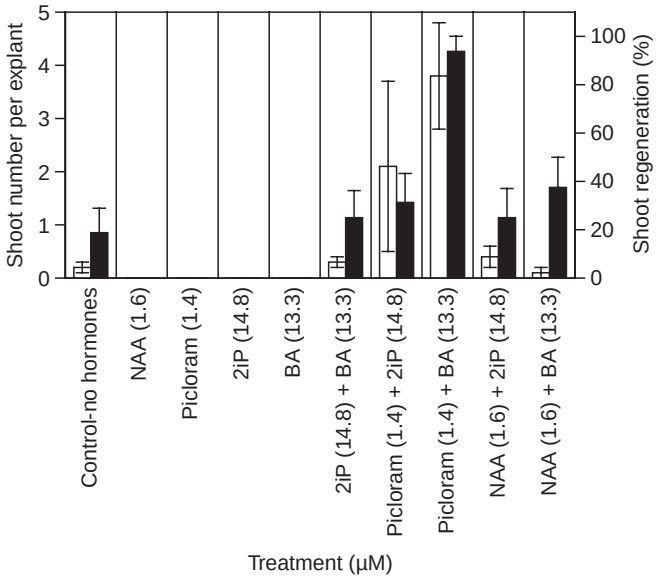

Fig. 1 Effect of plant growth regulator treatments on the regeneration and number of shoots in the garlic clone 'Piacenza' – explant age 5 months. *White bars* shoot number per explant, *black bars* shoot regeneration (%), *error bars* = ± SE

Regeneration from 12-month-old callus

Analysis of variance demonstrated that clone, treatment, and clone × treatment had significant effects on shoot number per explant ($F = 5.5$, $P = 0.0002$; $F = 11.4$, $P = 0.0001$; $F = 4.3$, $P = 0.0001$, respectively) and shoot regeneration ($F = 5.7$, $P = 0.0002$; $F = 14.3$, $P = 0.0001$; $F = 3.9$, $P = 0.0001$, respectively) in 12-month-old garlic callus. The significant interaction of clone × treatment indicated that not all plant growth regulator treatments stimulated diverse clones to regenerate with the same level of success. Block effects were not significant.

The optimum plant growth regulator treatment for shoot number per explant and shoot regeneration (%) was specific for different garlic clones (Fig. 2). 'PI383819' regenerated with most of the plant growth regulator treatments, while the other clones primarily regenerated only on the picloram plus BA treatments. The 1.4 μM picloram plus 13.3 μM BA treatment stimulated high shoot regeneration (%) for most clones and the 3.5 μM picloram plus 8.9 μM BA treatment was

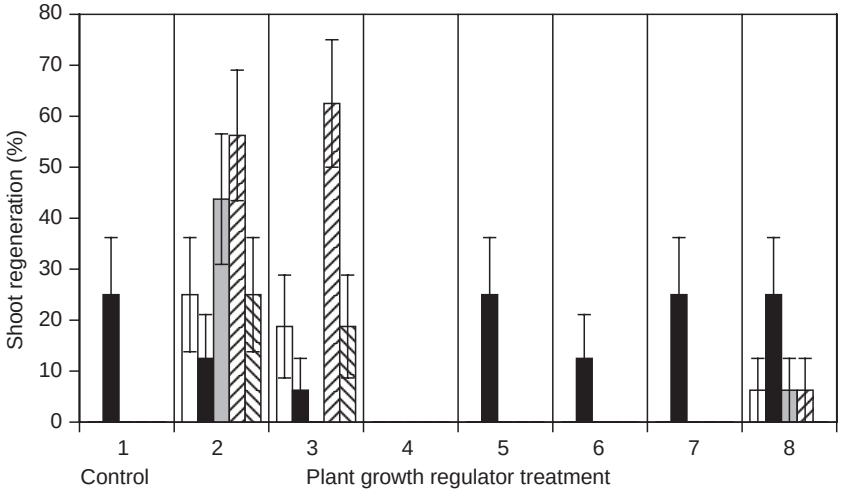

Fig. 2 Comparison of plant growth regulator treatments [*1* Control – no plant growth regulators, *2* picloram (1.4 μM) + BA (13.3 μM), *3* picloram (3.5 μM) + BA (8.9 μM), *4* TDZ (0.01 μM), *5* TDZ (0.025 μM), *6* TDZ (0.04 μM), *7* TDZ (0.075 μM) *8* kinetin (4.7 μM)] for shoot regeneration (%) in the garlic clones 'M/V' (*white bars*), 'PI383819' (*black bars*), 'Piacenza' (*gray bars*), 'RAL27' (*angled left bars*) and 'Creole Red' (*angled right bars*). Explant age: 12 months. *Error bars* = ± SE

comparable for all clones but 'Piacenza' (Fig. 2). Comparable trends in the effects of plant growth regulator treatments and garlic clones were observed for shoot number per explant as for shoot regeneration (%) (data not presented).

TDZ treatments promoted very little shoot regeneration in garlic (Fig. 2). In contrast, the cytokinin TDZ has been reported to promote the regeneration of recalcitrant crops such as large-seeded legumes (Mohamed et al. 1993) and garden leek with the subsequent production of healthy plantlets (Hong and Debergh 1995) at concentrations similar to those we used. Similarly, Kehr and Schaeffer (1976) reported that kinetin stimulated regeneration in garlic. However, in the regeneration of 12-month-old callus, the addition of kinetin to the basal medium promoted significantly less shoot production than the 1.4 μM picloram + 13.3 μM BA treatment.

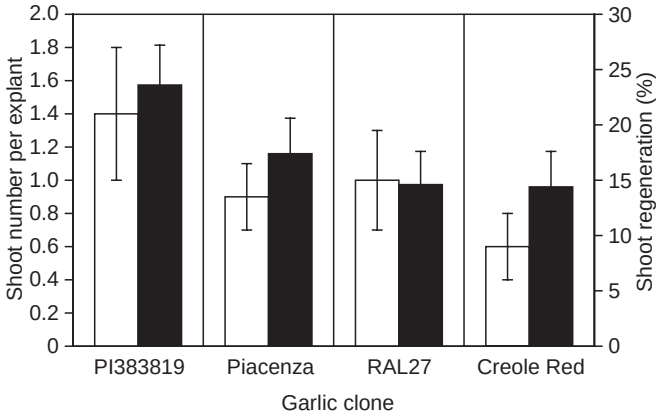

Fig. 4 Effect of garlic clone on the production of shoots in 16-month old explants. *White bars* shoot number per explant, *black bars* shoot regeneration (%), *error bars* = ± SE. Results presented represent the average of four plant growth regulator treatments

Regeneration from 16-month-old callus

All combinations of picloram plus BA comparably stimulated shoot production (18.6–24.3%). As in the regeneration of both 5- and 12-month-old callus, several of the growth regulator treatments were comparable, but the 1.4 μM picloram + 13.3 μM BA treatment was generally more effective in the regeneration of 16-month-old callus (Fig. 3). Clone 'PI383819' regenerated somewhat more shoots than any of the other clones tested, although undifferentiated callus from all four clones produced shoots after 16 months (Fig. 4). Roots were able to be produced on all garlic callus with regenerated shoots, and complete plants were readily produced.

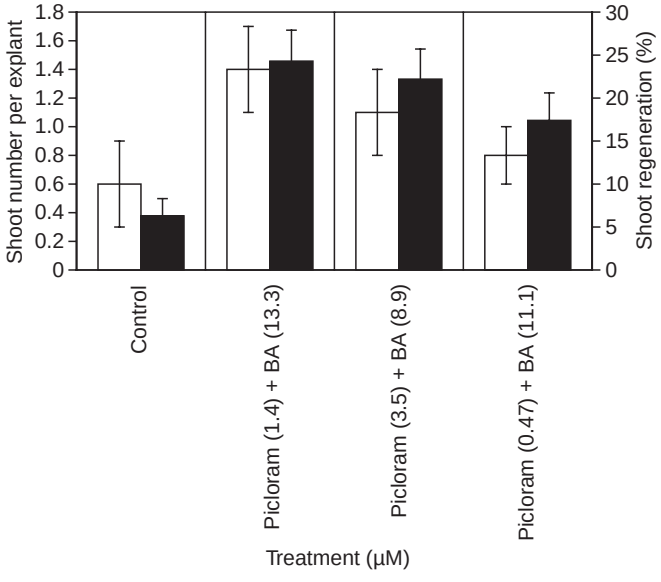

Fig. 3 Effect of plant growth regulator treatments on the production of shoots in 16-month-old garlic explants. *White bars* shoot number per explant, *Black bars* shoot regeneration (%), *error bars* = ± SE. Results presented represent the average of four clones: 'PI383819', 'Piacenza', 'RAL27' and 'Creole Red'

Conclusions

The plant growth regulator treatment to most reliably promote regeneration in long-term garlic callus cultures was the combination of 1.4 μM picloram plus 13.3 μM BA. With the regeneration of garlic callus, the production of shoots was the critical event needed to produce plantlets, since root growth was stimulated by transferring the plantlets to growth regulator-free medium (Myers and Simon 1998). Regeneration of each clone varied as a function of plant growth regulator treatments and the age of the callus. Other studies have also reported significant intraspecific genetic variation in the regeneration of *Allium* species (Phillips and Luteyn 1983; van der Valk et al. 1992).

The regeneration system described here may be useful for clonal propagation since diverse garlic clones can be maintained as callus for extended periods of time in culture while retaining their regeneration capacity. The procedure may also be useful for garlic transformation since the selection of putative transformed callus, like other aspects of garlic growth, is a slow process. The improved method of long-term regeneration of garlic callus might be useful in garlic improvement programs.

References

Abo El-Nil MM (1977) Organogenesis and embyrogenesis in callus cultures of garlic (*Allium sativum* L.). Plant Sci Lett 9:259–264

Ayabe M, Taniguchi K, Sumi S (1995) Regeneration of whole plants from protoplasts isolated from tissue-cultured shoot primordia of garlic (*Allium sativum* L.). Plant Cell Rep 15:17–21

Barandarian X, Martin N, Rodriguez-Conde MF, DiPietro A, Martin J (1999) Genetic variability in callus formation and regeneration of garlic (*Allium sativum* L.). Plant Cell Rep 18:434–437

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hong W, Debergh P (1995) Somatic embryogenesis and plant regeneration in garden leek. Plant Cell Tissue Organ Cult 43:21–28

Kehr AE, Schaeffer GW (1976) Tissue culture and differentiation of garlic. HortScience 11:422–423

Mohamed MF, Coyne DP, Read PE (1993) Shoot organogenesis in callus induced from pedicel explants of common bean (*Phaselous vulgaris* L.). J Am Soc Hortic Sci 118:158–162

Myers JM, Simon PW (1998) Continuous callus production and regeneration of garlic (*Allium sativum* L.) using root segments from shoot tip-derived plants. Plant Cell Rep 17:726–730

Nagasawa A, Finer J (1988) Induction of morphogenic callus cultures from leaf tissue of garlic. HortScience 23:1068–1070

Novak FJ (1980) Phenotype and cytological status of plants regenerated from callus cultures of *Allium sativum* L. Z Pflanzenzuecht 84:250–260

Novak FJ (1990) *Allium* tissue culture. In: Rabinowitch HD, Brewster JL (eds) Onion and allied crops, vol. 1. CRC Press, Boca Raton, Fla., pp 233–250

Pandey JG, Suprasanna P, Rao PS (1996) Tissue culture and differentiation of garlic (*Allium sativum* L.). Physiol Mol Biol Plant 2:179–182

Phillips GC, Luteyn KJ (1983) Effects of picloram and other auxins on onion tissue cultures. J Amer Soc Hortic Sci 108:948–953

Pooler MR, Simon PW (1993) Characterization and classification of isozyme and morphological variation in a diverse collection of garlic clones. Euphytica 68:121–130

SAS Institute (1982) SAS user's guide: basics 1982 edn. SAS Institute, Cary, N.C.

van der Valk P, Scholten OE, Verstappen F, Jansen RC, Dons JJM (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species. Plant Cell Tissue Organ Cult 30:181–191