Novel Method of Rapid Micropropagation using Cyclic Bulblet Formation from Root Tip Explants in Garlic

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Summary

A novel procedure for rapid and cyclic clonal propagation of garlic (*Allium sativum* L.) through *in vitro* bulblet formation was developed. In contrast to the previously employed micropropagation methods that make use of a single shoot tip, basal plate or bulb scale explant per clove, we have used more than 40 root tips per clove of the garlic 'White roppen' as starting material. Use of roots as explants gave the unique opportunity of a continuous bulblet formation. One cycle of the process was consisted of three steps: (a) shoot initiation from root tips cultured on MS medium containing 1 μ M NAA and 10 μ M BA, (b) shoot proliferation on MS medium supplemented with 0.5 μ M BA, and (c) bulblet formation on MS medium containing 12% sucrose. After completion of one cycle, *in vitro* roots were used as secondary explants. One cycle was completed within 4 months, and 316 bulblets were produced from the roots of one clove in the first cycle. This method has potential for the production of 5,838 bulblets from a single clove after two cycles completed within 10 months.

Key Words : *Allium sativum* L., clonal propagation, cyclic culture, *in vitro* roots, organogenesis, rapid multiplication, root apex culture.

Introduction

Cultivated garlic (*Allium sativum* L.) is a sexually sterile crop that is exclusively propagated vegetatively (Novak 1990). Its propagation rate in the field is 5 to 10 per year (Nagakubo *et al.* 1993). For rapid propagation, garlic relies on *in vitro* methods. *In vitro* propagation of garlic is mainly based on the formation of adventitious shoots regenerated from callus. However, callus cultures are reported to cause genetic instability and the efficiency of regeneration from degenerated callus is not high (Nagakubo *et al.* 1997). Micropropagation through multiple shoot formation from meristem tip with little or no callus formation has been reported by many authors (Bhojwani 1980, Osawa *et al.* 1981). Induction of multiple shoot directly from the meristem tip is preferred to adventitious shoot regeneration from callus culture. However, direct multiple shoot induction from garlic shoot tips was sporadic (Moriconi *et al.* 1990, Nagakubo *et al.* 1993). Shoot formation from the basal

plate and flower organs is also reported (Osawa *et al.* 1981, Suh and Park 1993, Mohamed-Yasseen *et al.* 1994).

Moreover, none of the previous methods is possible throughout the year due to the unavailability, dormancy, or storage diseases of the bulbs. Hitherto, a method to enable a continuous propagation for rapid multiplication has not been developed. In fact, no controlled embryo, shoot or bulb production system has been obtained from long-term tissue cultures in garlic. However, there is a real need of clones in economically important *Allium* crops like garlic and shallots, that are vegetatively propagated, as well as leek and onion for breeding programs (Kahane *et al.* 1992 and references therein).

Micropropagation through *in vitro* bulblet formation has been used as a reliable and excellent alternative to the plantlet formation. Unlike plantlets, bulblets need no acclimatization, can be stored using small space, are tolerant to desiccation, and are easy to handle and transfer. *In vitro* bulblet formation has been achieved with various explants including shoot tip, basal plate and bulb scale (Ravnikar *et al.* 1993, Nagakubo *et al.* 1993, Havranek and Novak 1973, Mohamed-Yasseen *et al.* 1994). However, the rate of multiplication is not sufficiently high for the quick multiplication of a new and quality cultivar and the time, in most cases, is very long. Moreover, excision of only one bulb scale, shoot tip or basal plate explant from a clove seems to be cost and labor intensive as well as time consuming. Therefore, use of these explants in the *in vitro* propagation through bulblet formation may not be an effective alternative to the traditional seed clove method of garlic propagation.

'White roppen' garlic produces more than forty roots per clove when sprouted aseptically. Therefore, roots could be an attractive source of explant and use of root tips appears to be cost and labor effective as well as less time consuming than use of any other explants. Unfortunately, the maximum rate of plant regeneration was very low (7 to 20%) in garlic root-apex derived calli (Shuto *et al.* 1993). However, recent recovery of adventitious shoots and plantlets from root tips in garlic cv. 'White roppen' has been proved very efficient (Haque *et al.* 1997). *In vitro* bulblet formation from root tips has a tremendous potential for repeated use of the tips of the roots that develop on the shoot proliferation and bulblet formation media. The bulblets after harvest leave large number of roots. If those roots could be effectively used for shoot induction and bulblet formation, there would be no need of additional use of new explants and the repeated use of those roots could give a continuous

year-round system of micropropagation in garlic for the first time. Bulblets, after harvest, can be transferred to the field immediately or after storage. Therefore, the objective of the present study was to develop a protocol of continuous bulblet formation from garlic root tips to produce rapidly true-to-type propagules and ensure micropropagation over a long period.

Materials and Methods

Garlic (cv. 'White roppen') bulbs stored at 4 °C were separated into cloves and outer, dry, papery bulb scales were removed. Healthy cloves were surface-sterilized by placing in 70% ethanol for 30 sec and 0.1% sodium hypochlorite solution, containing 2–3 drops of Tween 20 per 100 ml, for 20 min with frequent agitation, and washed three times for at least 2 min in sterile distilled water. The cloves were sprouted aseptically on sterile water–agar (0.7% agar in distilled water) contained in culture bottles (5.7×10.2 cm) closed with polypropylene leads and sealed with parafilms. Within 10 to 15 days, the cloves developed into plantlets with profuse roots that were used as primary explants in this study (Fig. 1a).

Root tips (2–3 mm) from plantlets 10–15 days after sprouting were excised aseptically and placed horizontally on MS (Murashige and Skoog 1962) medium containing 1 μ M NAA and 10 μ M BA for shoot initiation (Haque *et al.* 1997). Five explants were cultured per bottle. After 30 days, the explants that had initiated shoots were subcultured on MS medium supplemented with 0.5 μ M BA for proliferation. Multiple shoots that developed within about 40 days were separated into single shoot. The individual shoots were cultured for about 2 months on growth regulator-free MS medium containing various concentrations of sucrose for bulblet formation. Multiple shoot clumps were also cultured on medium containing 12% sucrose.

Root tips were also excised from roots produced on proliferation and bulblet formation medium (Fig. 1b) to test their efficacy for regeneration and bulblet formation. *In vitro* root tips (secondary explants) were cultured on the same medium, MS medium supplemented with 1 μ M NAA and 10 μ M BA. To overcome a problem with the shoot proliferation in case of roots from proliferation and bulblet formation medium, the initiated shoots were transferred to MS medium with 6% sucrose, before transfer to the bulb formation medium. For bulblet formation, the multiple shoot clumps were cultured for 2 months on MS medium containing 12% sucrose.

In all tests, MS medium was supplemented with 0.8% agar (INA, BA-30) and 3% sucrose, except for the experiments with bulblet formation. The media pH was adjusted to 5.8 before the addition of agar. The media was autoclaved at 121 °C for 20 min. Forty ml medium was used per culture bottle. Twenty explants were cultured per treatment and the treatments were repeated three times. The culture bottles were closed with poly-

Fig. 1. Root tip explant sources and shoot clumps induced from them.

- (a) Primary explants from sprouted clove (7 days after sprouting). Garlic clove was sprouted aseptically on 0.7% agarwater.
- (b) Secondary explant source, the roots from the bulblet formation medium after 50 days of the transfer of the multiple shoots to the medium.
- (c) Multiple shoots from the primary explant (bar=1 cm).
- (d) Proliferated shoots from a secondary explant from bulblet formation medium (bar=1.7 cm).

propylene leads and sealed with parafilms. All cultures were incubated at 28 ± 1 °C with continuous light ($15 \mu \text{Em}^{-2}\text{s}^{-1}$) provided with cool white fluorescent light. The data were analyzed using ANOVA and t-test or Duncan's Multiple Range Test (DMRT). Bulblets were cultivated in the planters inside glasshouse

Results

Shoot initiation and proliferation

Within one month, direct shoot formation occurred in 93.3% of the primary explants cultured on the shoot initiation medium (Table 1). The frequency of shoot induction on root tips excised from the multiple shoots before they were transferred to the bulblet formation medium was 48.3% while that with the explants from the bulblet formation medium, after the harvest of the bulblets, was 78.3%. It revealed a difference in the morphogenetic response between sprouted clove derived root and *in vitro* root. Although there was a slightly

Table 1. Effect of explant source on regeneration and bulblets formation

Root tip explant source	% Shoot ¹⁾ initiation	No. of shoot ²⁾ per explant	No. of bulblet ³⁾ per explant	No. of root ³⁾ per explant
Sprouted clove	93.3 ± 2.88 a	10.6 ± 3.50 a	7.8 ± 2.32 a	28.2 ± 5.15 ab
Shoot proliferation medium	48.3 ± 2.89 c	5.0 ± 1.67 b	3.6 ± 1.36 b	22.6 ± 6.37 b
Bulblet formation medium	78.3 ± 2.88 b	9.8 ± 2.71 a	6.2 ± 1.72 a	35.6 ± 7.17 a

¹⁾ On MS medium containing 1 μ M NAA and 10 μ M BA.

²⁾ On MS medium supplemented with 0.5 μ M BA.

³⁾ On growth regulator free MS medium containing 12% sucrose.

Mean values in each column followed by different alphabets are significantly different at 5% level by Duncan's Multiple Range Test (DMRT).

lower regeneration capacity of the *in vitro* roots, it is of great importance in the *in vitro* propagation of garlic due to the prolific production of roots *in vitro*. There was no change in the mode of regeneration. Like the sprouted clove derived root tips, the *in vitro* root tips regenerated without any callus formation. Primary explants, that had no shoot induction produced callus. So far secondary explants are concerned, the explants which failed to regenerate shoot either formed callus or died on the medium.

Shoot proliferation on MS medium containing 0.5 μ M BA was effective for the explants from sprouted cloves (Fig. 1c). Initial observation on the proliferation of shoots initiated in root tips from multiplication and bulblet formation medium (*in vitro* root tips) was discouraging. There were considerable number of shoots that were small and seemed ineffective for bulblet formation. Only one shoot became well developed rendering others smaller. In our preliminary experiment, higher sucrose promoted shoot growth. We tested that growth regulator free MS medium with 6% sucrose and found better proliferation (Fig. 1d). Maximum number of shoots was found with primary explants, followed by the secondary explants from bulb formation medium. Least number of shoots was evident with the secondary explants from the shoot proliferation medium (Table 1). The *in vitro* root tip derived shoots grew slowly compared to the primary explant derived shoots. Therefore, it took longer time for the proliferation of secondary explants derived shoots. Shoots from primary explants proliferated within 40 days while shoots from secondary explants took about 2 months to be suitable for transfer to the bulblet formation medium.

Bulblet formation

In vitro bulblet formation is promoted by high osmoticum mainly induced by higher concentration of sucrose. However, there is little information on the appropriate concentration of sucrose for bulblet formation. We tested various concentrations of sucrose to find out the most effective one using single shoots from primary explants. Bulbing was promoted by all the higher sucrose concentrations. Bulblet formation was evident within 40 days of culture (Fig. 3a). But the

Fig. 2. Effects of various sucrose concentrations on bulblet weight, diameter and length. Bars followed by same letters do not differ significantly at 5% level by Duncan's Multiple Range Test (DMRT). There was no bulblet formation on the medium containing 3% sucrose.

bulblet size differed among the concentrations (not significant). The best concentration of sucrose was 12% (Fig. 2) below which bulblet formation was poor and above which bulblet formation was slightly suppressed. Bulblet fresh weight, diameter and length was higher on the medium containing 12% sucrose compared to those containing 8% and 16% sucrose (Fig. 2). No bulblet formed on the medium supplemented with 3% sucrose. We considered a bulblet when it weighed above 50 mg. There was bulblet formation from 100% shoots cultured on the media containing 8–16% sucrose.

Multiple shoot clumps from primary explants were transferred to the bulblet formation medium, MS medium with 12% sucrose to compare with the individual shoots that produced bulblets in our earlier experiment (Fig. 3b). They produced bulblets on this medium (Fig. 3c). Compared to the single shoot the multiple shoots had average smaller sized bulblets (Table 2). We preferred the bulblet formation from the multiple shoots as they are easy to transfer, less laborious and there is no loss of the smaller shoots. The multiple shoots can adjust themselves quickly with the medium because they contain roots and there is no injury that can happen

Table 2. Comparative bulblet formation from single and multiple shoots

Type of shoot	Bulblet weight (g)	Bulblet dia meter (cm)	Bulb length (cm)	Neck dia meter (cm)	Bulbing ratio
Single	0.6 ± 0.4	0.92 ± 0.18	1.24 ± 0.22	0.20 ± 0.05	0.22 ± 0.03
Multiple	0.4 ± 0.1	0.78 ± 0.04	1.00 ± 0.05	0.12 ± 0.01	0.15 ± 0.01
Significance	ns	p<0.05	p<0.05	p<0.05	p<0.05

ns=not significant by t-test.

Single or multiple shoots were cultured on MS medium containing 12% sucrose.

Cultures were maintained in continuous light at 28 °C.

Fig. 3. *In vitro* bulblet formation from root tip explants in garlic.

- Formation of bulblet on medium containing 12% sucrose (bar=1.3 cm).
- Bulblets induced on single shoot.
- Bulblets formed from the multiple shoot clumps from primary explants (bar=1 cm).
- Bulblets formed from a secondary explant. Multiple shoot clumps, either separated into single or without separation, were cultured on MS medium containing 12% sucrose. The cultures were incubated in continuous light ($15 \mu\text{Em}^{-2}\text{s}^{-1}$) at 28 °C.

during separation of the multiple shoots into single.

Secondary explant derived shoots produced bulblets on the same medium described for the primary explants. However, the size of the bulblets, the percentage of shoots produced bulblets and the time required for bulblet formation varied. Bulblets induced with the secondary explants from bulblet formation medium were similar to those from the primary explants (Figs. 3c & 3d). Highest number of bulblets was produced by the primary explants followed by secondary explants from bulblet formation medium and shoot proliferation medium, respectively (Table 1). Percentages of shoots with

bulblets weighing above 50 mg in roots from sprouted clove, shoot multiplication medium and bulblet formation medium were 74, 72 and 63, respectively. Bulblet formation from *in vitro* root tips required longer time. Bulblet formation was completed within 2 months in *in vitro* roots while that required at least 2 weeks shorter time in clove derived root tips.

Roots available for next cycle

After harvest of the bulblets, there was huge number of roots that developed during the bulblet formation and was available for excision in the next cycle. There was little difference in the number of roots formed due to variation of the explant source. However, the highest number of roots were produced from the explant from bulblet formation medium (Table 1). We are continuing further study with these roots. In the third stage, the response was similar to the second stage.

Transfer of the bulblets to soil

Bulblets were successfully transferred to the soil immediately after harvest or after storage at 4 °C for few months. They produced normal plants in both conditions. There was 100% sprouting for large and medium sized bulblets. Small bulblets had 90% sprouting. The immediate sprouting of the *in vitro* bulblets indicates the presence of no dormancy. The successful sprouting of the bulblets after storage reveals a wide applicability of the process over the entire year. Bulblets can be produced whole the year and planted in the soil during the next available season after storage.

Calculation of multiplication

We excised 43.6 explants per clove. There was shoot formation on 40.5 explants (93.3%). In the first cycle 316 bulblets were harvested from 429 shoots within 4 months. There was 1,142 roots available after the first cycle (Table 3). We excised root tips from these roots. Although we could not use all roots at a time due to limitation of space, it was estimated from the available data that in the second cycle, 5,522 bulblets could be produced from 8,729 shoots within 6 months rendering 31,712 roots for the next cycle. Thus, within 10 months the net multiplication is 5,838 (Table 3). The number of bulblets and the roots for next cycle will be increased logarithmically.

Table 3. Possible multiplication rate of garlic by cyclic culture of root tips

Culture step	Initial explant number	Explant regenerated	Shoot produced	Bulblets harvested	Roots formed	Time required
First	43.4	40.5	429.4	315.9	1142.0	4 months
Second	1142.0	890.8	8729.0	5522.0	31712.0	6 months
Total	1185.4	913.3	9158.4	5837.9	32854.0	10 months

Calculated on the basis of 43.4 roots per explant, 93.3% shoot initiation, 7.8 bulblets per explant, and 28.2 roots per explant in the first cycle, while 78.3% shoot initiation, 6.2 bulblets per explant, and 35.6 roots per explant in the second cycle (see Table 1).

Fig. 4. Diagrammatic representation of cyclic clonal propagation of garlic through bulblet formation from root tip explants.

Discussion

This protocol gives an opportunity of cyclic bulblet formation (Fig. 4), which is not possible with shoot tip, bulb scale or basal plate culture. Bulblet formation from root tip explants is preferable to our previous method of plantlet formation (Haque *et al.* 1997), which needed acclimatization of the plantlets and failed to give a continuous system through the repeated use of the *in vitro* roots. Each cycle completed within 4–6 month-period and thereby, at least two cycles are possible in a year. This system has therefore, potential use for rapid multiplication of superior or high-yielding and disease resistant genotypes.

Shoot regeneration occurred without a callus phase. This is an important criterion in micropropagation industry to maintain genetic stability that is not possible in the callus cultures of garlic (Novak *et al.* 1986, Maggioni *et al.* 1989). Direct shoot regeneration is preferred to

regeneration that includes a callus formation. However, further studies are required to confirm the genetic fidelity of the bulblet derived plants.

Percentage of shoot induction varied with the source of explant in this study and explants from sprouted clove had higher frequency than that from *in vitro* roots. This difference might be due to the difference in the quality of the roots. The *in vitro* roots were thinner than the clove derived roots. However, regeneration from more than 78% *in vitro* roots is a great achievement in garlic tissue culture and is sufficient for practical use to obtain enough amount of virus-free garlic plants because of high rate of *in vitro* root formation (Shuto *et al.* 1993).

Bulblet formation occurred on media supplemented with 8 to 16% sucrose in this study and a medium containing 12% sucrose was optimum. There have been reports of bulblet formation, which is influenced by a variety of factors (Abo El-Nil 1977, Bhojwani 1980, Nagakubo *et al.* 1993, Seabrook 1994, Mohamed-Yasseen *et al.* 1994, Zel *et al.* 1997). With shoot tip

explants of the same cultivar, Nagakubo *et al.* (1993) reported that bulblets were formed after a low temperature treatment of 6 months followed by culture on LS medium with 6 to 12% sucrose. The use of mannitol and glucose in combination with a low salt concentration promoted bulblet formation in shoot cultures of other cultivars (Seabrook 1994). However, this medium was not effective in the present cultivar (personal observation). Mohamed-Yasseen *et al.* (1994) reported that 12% sucrose in combination with 0.5% activated charcoal induced bulblet formation from shoot tips. Recently, Zel *et al.* (1997) reported that 8% sucrose significantly increased the percentage of bulblet formation and a simultaneous use of jasmonic acid and sucrose can improve bulblet formation. Compared to the above reports, the present method is the simplest and induce bulblet formation only by high sucrose concentration. Similar result has been reported for onion also (Mohamed-Yasseen and Splittstoesser 1992).

Another important feature of this study was the light and temperature conditions of the culture. In the previous studies, culture conditions of 25 °C temperature and 16L/8D light were used most commonly for bulblet formation (see for review Nagakubo *et al.* 1997). We maintained our culture in a continuous light condition at 28 °C temperature. It is apparent that bulblet formation occurs more rapidly under conditions of longer exposure to light and a higher temperature and using lower temperature and shorter light duration reduced the efficiency of bulblet formation (Nagakubo *et al.* 1997). However, there is no report of using a constant light and more higher temperature for the bulblet formation in the past. Therefore, the continuous light and 28 °C temperature used in this study might be appropriate conditions for *in vitro* bulblet formation in garlic. However, further study is needed to confirm it.

In our study, the bulblet weight, diameter and length are much higher than the previous reports. We did not consider a bulblet unless its weight was more than 50 mg. The bulblet weight ranged from 50 mg to 1.569 g and the average being 0.6 g for single shoot and 0.4 g for multiple shoots. Very few reports mentioned the average bulblet weight that ranged from 53.9 mg to 500 mg among different cultivars (Nagakubo *et al.* 1993, Seabrook 1994). Bulblets of higher diameter and length than the previous reports were found in this study. Only 2 to 6 mm diameter of the bulblets was recorded earlier (Seabrook 1994, Zel *et al.* 1997). Therefore, a better bulblet formation is reported here. This differences might be due to the differences in the cultivar, explant and the culture conditions used.

We preferred culturing multiple shoot clumps over separated shoots (Nagakubo *et al.* 1993, Mohamed-Yasseen *et al.* 1994) to avoid a lot of additional hand work and to simplify the process (Zel *et al.* 1997). It also avoids a possible injury to the shoots and loss of small shoots. Average 7.8 bulblets were regenerated per multiple shoot clump per explant from sprouted clove. A

varied multiplication rate per explant has been reported from other explants. There was a wide variation in the culture period that ranged between 2 and 15 months (see Nagakubo *et al.* 1997). Average 12 microbulbs were achieved from one basal plate explant per clove after 3 months of culture (Zel *et al.* 1997). Nagakubo *et al.* (1993) were able to produce 640 bulblets from one shoot tip in 16 months. Intact shoot tips produced only 59 bulblets per 50 explants (Seabrook 1994), whereas sliced shoot tips produced 8.2 bulblets per explant (Mohamed-Yasseen *et al.* 1994). On the contrary to the use of only one shoot tip, basal plate or bulb scale explant per clove in the previous methods, we could excise 43 root tips per clove. In our study, 316 bulblets were harvested from the root tips of a single clove within 4 months of the first cycle. In additional one cycle of next 6 months, 5,522 bulblets could be produced from the roots that remained after the harvest of the bulblets in the first cycle. It is a significant achievement in the garlic micropropagation.

The bulblets were successfully transferred to the soil and depending on size, 90 to 100% of them formed plants. No dormancy was observed as the bulblets sprouted immediately after harvest, and even sprouted in the culture when the harvest was delayed. This finding is in contradiction with that of Nagakubo *et al.* (1993) and in agreement with the result of Mohamed-Yasseen *et al.* (1994). The bulblets could be stored several months without any effect in their ability to sprout and to form plant.

The present protocol is promising for increasing the number of explant per clove and hence the number of bulblets regenerated per clove, shortening the culture period and accelerating the rate of multiplication. It is cost and labor effective. The commercially cultivated garlic is sexually sterile and propagated by seed cloves. The method described here can be effectively used for the continuous production and supply of garlic bulblets, thus can substitute the seed clove method of garlic propagation. Once culture is established, there is no need of additional explant. We propose this technique for the commercial micropropagation of garlic.

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