Effect of explants type and culture medium on *in vitro* callus induction and shoot regeneration in *Allium chinense*

BAH Amadou Apho [1, 2]*, Malle Abdou[2], Kansaye Laya[2] kansaye Aly[2]
[1]Zhejiang University, College of Agriculture and Biotechnology,
China
[2]Institut Polytechnique Rural de Formation et de Recherche Appliquée IPR/IFRA
Katibougou- MALI
* Corresponding author's email address : aphobah@yahoo.fr

ABSTRACT: The present investigation was undertaken to develop a protocol for rapid callus initiation and plant regeneration of *A. chinense*. Explant type such as root, leaf and bulb were used in order to test their potential on callus induction. They were cultured on different media (B5, MS and LS) containing various concentrations of different growth regulators to determine the appropriate culture media and the suitable explant type promoting callus formation . It was found that basal plate of bulb proved to be the suitable explant for callus induction when cultured on the culture medium supplemented with 0.1 mg/L BA and 1.0 mg/l 2,4-D. The B5 medium containing1.5 mg/l 2,4-D promoted the best growth and proliferation of callus. Shoot regeneration occurred rapidly and vigorously when callus were transferred on B5 supplemented with 0.1 mg/l BA and 1.0 mg/l NAA.

Keywords: *Allium chinense*, Explant, Callogenesis, culture medium.

I. INTRODUCTION

Allium chinensis G. Don belongs to the family of *Alliacea.* It has been used also as spice food, and as Chinese folk medicine for the treatment of range of maladies such as heart problems, headache and worms for over 2000 years [1]. Because of its sterility, the plant is generally propagated vegetatively. Therefore, asexual propagation has a low multiplication rate and can spread viral infections, such as rakkyo strain of tobacco mosaic tobamovirus (TMV-R) [2], garlic latent virus (GLV) and onion yellow dwarf potyvirus (OYDV) [3]. The method of *in vitro* tissue culture is a practical option to ameliorate the effectiveness of multiplication and to remove viruses from infected crops. Thus, this approach can promote conventional *A. chinense* proliferation programs and create germplasms with higher yield, tolerance and resistance to viral diseases. Production of shoot *via* callus culture is one of potential approach to accomplish *A. chinense* genetic progress, because it has some benefit of shoot induction *via* callus induction over direct shoot formation. Callus stage is an important way in tissue culture methods. It aims to induce variability and promotes new desirable characters such as pest resistance in *Allium* plants [4]; [5]. Furthermore, callus induction is also an essential phase for protoplast fusion, a valuable method in genetic enhancement of vegetatively generated *Allium* species for insertion functional genes or introduction new elite cultivars [5]; [6]. Earlier studies have been reported on callus formation and shoot proliferation of *Allium* species [7]; [8]; [9]; [10]; [11]; [12]; [13]; [4]; [14]. Callus initiation and shoot formation were promoted by the effect of growth regulators [13]; [15]; [16]; [17]. It was also observed shoot formation on some types of callus during the phase of callus induction [18]; [16]; [19a].

This research intended to determine a practical method of *in vitro* tissue culture which could supply a useful system for callus induction and plant regeneration of *A. chinense*.

II. MATERIALS AND METHODS

2.1 Plant Materials

Plants were collected from the Experimental Station of Zhejiang University.

2.2 Explant Preparation

Explants (basal plates, roots and young leaves), were washed under running tap water for 1 h and soaked with mild detergent solution for 10 min. Then, they were washed three times with distilled water. The explants were surface sterilized and soaked in 75% ethanol for 5 minutes and followed by 0.1% HgCl2 for 15 minutes. These disinfected fragments were then rinsed three times with sterile distilled water. The explants were excised into pieces of 2 to 3mm and aseptically transferred onto a sterile to culture medium. All works were done under sterilized condition in a laminar air-flow cabinet

2.3 Culture Medium

Explants cut into small pieces were cultured on MS (Murashige and Skoog, 1968), B5 (Gamborg, 1968) and LS (Linsmaier and Skoog) supplemented with 30 g/l sucrose, 7 g/l purified agar, and various concentrations of growth regulators. The pH for each medium was adjusted to 5.8, and was autoclaved at 121°C for 20 minutes.

All cultures were inoculated at 25± 1º C under a photosynthetic photon flux density (PPFD) of 20µ mol m^2s^1 provided by cool white fluorescent tubes with a photoperiod of 14/10 h (day/night).

2.4 Callus Induction

The disinfected explants were excised and cultured on different medias (MS, B5 and LS) supplemented each with 1.0 mg/l 6-benzyladenine (BA) and 1.0 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), in order to determine and identify the suitable explant and the appropriate culture medium inducing callus. It was found that basal plate of bulb was the most responsiveness explant inducing callus while leaf and root were less active. The use of B5 was more efficient than MS and LS. Hence, the following experiments were performed using basal plate of bulb as explant and B5 as basal medium.

2.5 Growth Regulators

The pieces of basal section of bulb were inoculated on B5 media containing the combination of various concentrations of growth regulators BA (0.1, 0.5, 1.0, 2.0, 3.0 mg/l), NAA (0.1, 0.5, 1.0, 2.0, 3.0 mg/l) and 2,4-D (0.1, 0.5, 1.0, 2.0, 3.0 mg/l), in order to identify the best growth regulators combination promoting callus induction. It was found that the intermediate level of 2,4-D (1.0 mg/l) showed the highest frequency of callus induction after eight weeks of culture.

2.6 Callus Proliferation

The growth and the frequency of proliferation of callus derived from basal plate were evaluated. Previous experiments indicated that callus formation was inhibited with the presence of NAA in the medium. Consequently, for an improved growth of callus, media composition without NAA was tested. Callus with excellent growth were selected, cut into small pieces and were transferred to the medium consisted with 2,4-D (0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) and to the medium containing 1.0 mg/l 2,4-D with combinations of BA (0.1, 0.5, 1.0, 2.0 mg/l) . Subsequently, subculture was performed on the same medium conditions as mentioned above. Callus growth was recorded after up to 3 subculture passages. Callus was grown rapidly and vigorously on the medium containing BA (0.5, 1.0 mg/l) or 2,4-D (1.0 mg/l).

2.7 Adventitious Shoot Formation

After three subculture passages, callus with good growth ability were transferred to the medium supplemented consisted with different combinations of BA (0.1, 0.5, 1.0, 2.0, 3.0 mg/l) and NAA (0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), in order to determine the suitable growth regulators combination promoting adventitious shoot formation. It was revealed that the culture medium containing 0.1mg/l BA+1.0 mg/l was more efficient. The relative higher concentration of NAA inhibited shoot formation. Although, adventitious shoot formation occurred mostly on all media tested. Rooting was mostly observed on the medium containing 0.1mg/l BA +2.0 mg/l NAA.

2.8 Statistical Analysis

Data were analyzed statistically using the Statistical Analysis System program (SAS, 2001). The mean values were calculated and were compared by Duncan's multiple range tests ($P < 0.05$).

III. RESULT AND DISCUSSION

3.1 Effect of Explants Type on Callus Induction

The use of different type of explant affected significantly callus initiation in *A. chinense*. Callus was formed on all explants tested. However, basal plate was the most responsiveness with a rate 46.7% callus induced after eight weeks while roots and leaves showed a significantly lower rate of callus formation (11.5 and 1.3% respectively) (Figure 1**)**. This result indicated that basal plate was the most suitable explant for *Allium chinense* callogenesis. Similar result was reported in garlic by Luciani *et al*. [11].

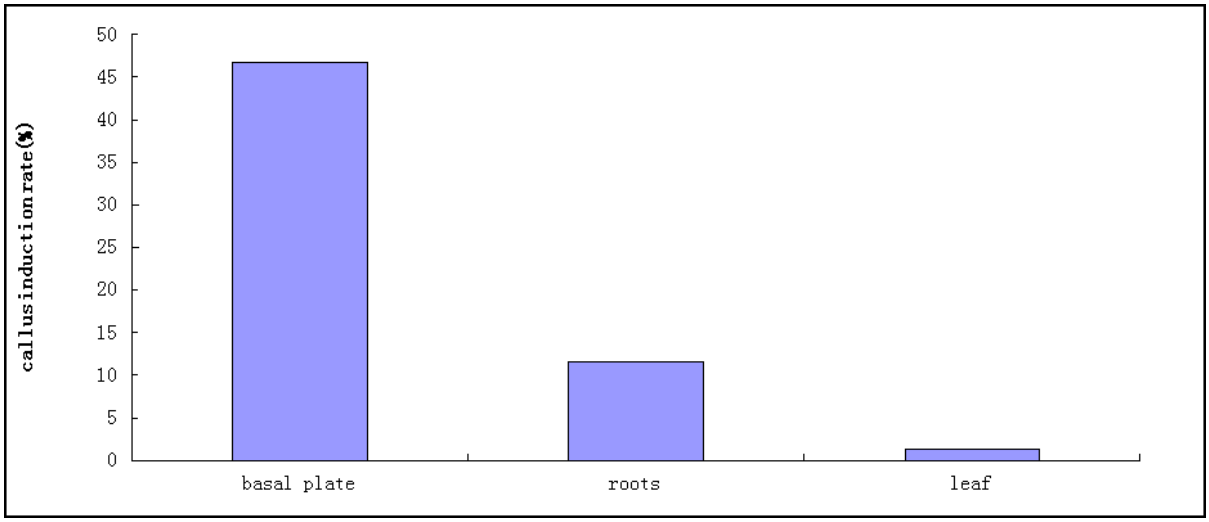

Figure 1. Effect of explant sources on callogenesis ability in *Allium chinense*

Callus induction from various explants after 8 weeks of culture in B5 medium containing 1.0 mg/l BA and 1.0 mg/l 2,4-D. values represent the average of 3 replicates.

3.2 Effect of Culture Medium on Callus Induction

The type of culture medium also has affected greatly callus induction. The highest rate of callus formation (44.6%) was obtained when explants were cultured on B5 medium, while MS and LS media appeared to be less effective with 32.1 and 13.2%, respectively ((Table 1). These results were similar to the data reported by Myers and Simon [13] who established the efficient of B5 on callus formation in *A. sativum* using basal plate as explant. The reason of the dissimilarity of callus induction on different culture media may be related to the differences of $NO3^{-}$/NH4+ ratio, an important factor on nitrogen uptake and pH regulation during plant tissue culture [20]. A lower $NO3^{-}$/NH4+ ratio in B5 medium have stimulated callus induction and growth from other *Allium* species [21]; [22]; [23]. Thus, we found that B5 was the best culture medium promoting callus induction in jiaotou, despite that successful callus induction has been observed on other growing media

Table 1. Effects of culture media on callus formation in *A. chinense*

Culture Medium	Number of explants	*Induction rate (%)*
MS	125	32.1
B5	100	44.6
LS	110	13.2

Data were recorded after 8 weeks of culture. Different medium contained 1.0mg/L BA and 0.5mg/L2,4-D.

3.3 Effect of Growth Regulator Combinations on Callus Induction

The combination of cytokine BA with auxins 2,4-D and NAA in the medium has remarkably affected callus induction (Table 2). The use of 0.1 mg /l BA with 1.0 mg/l 2,4-D proved to be the best growth hormones combination that stimulate callus induction, and 59.6% of explant has induced callus. Callus formation lowered as BA concentration increased when combined with 1.0 mg/l 2,4-D. The suppressive effect of higher concentrations of 2,4-D on callus induction was observed in the medium containing 0.1 mg/l BA+3.0 mg/l 2,4D.

The medium containing 0.1mg/l BA+1.0 mg/l NAA promoted callus induction at a rate of 37%. Therefore, we confirmed that the use of 2,4-D is more efficient than NAA in jiaotou callus formation. Additionally, in this experiment we have identified morphologically two kinds of callus by direct observation. The friable callus with whitish yellow color (easy to break in small pieces) and the compact callus with globular structure were observed

Table 2. Effects of different growth regulator combinations on callus induction

Growth regulator (mg/l)			Callus induction rate (%)
BA	2.4-D	NAA	
0	0.1	0	0
0	1.0	0	36
0.5	0	0	8
0.1	1.0	0	59
3.0	1.0	0	10
0.1	0	0.5	14
0.1	0	1.0	37
0.1	0	3.0	0

Data were recorded after 8 weeks of culture. Basal plates were inoculated on B5 basal media.

3.4 Effect of Growth Regulators on Callus Growth

After several subculture passages, it was observed an improved growth and changes on callus. Noticeable and rapid callus growth occurred when 2,4-D concentration increased from 1.0 up to 1.5 mg/l. The effectiveness of 2,4-D on callus proliferation was mostly observed in all the media tested. It was found that the use of 1.5 mg/l 2,4-D

affected more positively callus growth (Figure 2). Lower (0.5 mg/l) and higher concentration (2.5 mg/l) of 2,4-D suppressed callus proliferation. BA at either 0.5 or 1.0 mg/L combined with 1.0 mg/l 2,4-D also showed a perceptible callus growth and proliferation. However no apparent growth have been observed on the medium containing 2.0 mg/l BA + 1.0 mg/l 2,4-D (Table 3). In addition, we observed that proliferated callus were mostly light yellowish and friable. It was concluded that the auxine 2,4-D has a significant effect on callus growth in *A. chinense*. Similar effects of 2,4-D have been detected in garlic callus culture by Luciani *et al*. [11].

Figure 2. Callus growth and proliferation in B5 medium containing 1.5 mg/l 2,4-D

Table 3. Effects of growth regulators on callus proliferation

Growth regulators (mg/l)		observations
BA	2,4-D	
0	0	Non proliferation, non apparent change on callus
0	0.5	Unapparent proliferation, relatively small granules
0	1.0	Noticeable growth
0	1.5	Rapid proliferation
0	2.0	Unapparent proliferation
0	2.5	Unapparent proliferation
0.1	1.0	Unapparent proliferation
0.5	1.0	Apparent proliferation
1.0	1.0	Noticeable proloiferation
2.0	1.0	Unapparent proliferation

3.5 Effect of Growth Regulator Conbinations on Adventitious Shoot Formation.

Callus exhibiting good growth were selected and transferred to B5 media supplemented with different BA and NAA concentrations to evaluate their ability to regenerate adventitious shoots. The highest rate (85.85%) was observed when callus were transferred on the medium containing 0.1 mg/l BA and 1.0 mg/l NAA. The less

Table 4. Effect of growth regulator combinations on adventitious shoot regeneration from callus

Growth regulators (mg/l)		Shoot induction (%)
BA	NAA	
0.1	0	0
0.1	0.1	29
0.1	0.5	44
0.1	1.0	85
0.1	1.5	48
0.1	2.0	30
0.1	2.5	25
0.1	3.0	9

Data were recorded after 8 weeks of culture. Callus was inoculated on B5 basal media.

Effectiveness growth hormones combination affecting shoot formation was 0.1 mg/l BA +3.0 mg/l NAA on which 9.98% shoot were formed (Table 4). Concerning adventitious root formation, the results revealed that the combination of 0.1mg/l BA with 2.0 mg/l NAA was more efficient.

IV. CONCLUSION

According to the results obtained from this present study, it was found that explant type, culture medium and the growth hormone combination affected significantly *A. chinense in vitro* regeneration. Successful callus induction and plant regeneration is this plant could be achieved using basal plate as explant and B5 as suitable culture medium. The use of cytokine (BA) and auxins (2,4-D and NAA) had a significant effect on *A. chinense* callogenesis and shoot formation. Plant regeneration *via* callus culture could be a practical and valuable tool to improve the breeding program of *Allium chinense*.

REFERENCES

[1] Mann LK, Stearn WT (1960) Rakkyo or chiaotou (*Allium chinense* G. Don, syn. A. bakeri Regel) a little known vegetable crop. *Econ Bot* 14: 63–83

[2] Chen J, Watanabe Y, Sako N, Ohshima K, Okada Y (1996) Complete nucleotide sequence and synthesis of infectious in vitro transcripts from a full-length cDNA clone of a rakkyo strain of tobacco mosaic virus. *Arch Virol* 141: 885–900

[3] Sako I, Nakasone W, Okada K, Ohki S, Osaki T, Inouye T (1991) Yellow streak of rakkyo (*Allium chinense* G. Don), a newly recognized disease caused by garlic latent virus and onion yellow dwarf virus. *Ann Phytopathol Soc Japan* 57: 65–69

[4] Mukhopadhyay MJ, Sengupta P, Mukhopadhyay S, Sen S (2005) *In vitro* stable regeneration of onion and garlic from suspension culture and chromosomal instability in solid callus culture. *Sci Hort* 104: 1–9

[5] Fereol L, Chovelon V, Causse S, Triaire D, Arnault I, Auger J, Kahane R (2005) Establishment of embryogenic cell suspension cultures of garlic (*Allium sativum* L.), plant regeneration and biochemical analyses. *Plant Cell Rep* 24: 319–325

[6] Yamashita K, Hisatsune Y, Sakamoto T, Ishizuka K, Tashiro Y (2002) Chromosome and cytoplasm analyses of somatic hybrids between onion (*Allium cepa* L.) and garlic (*A. sativum* L.). *Euphytica* 125: 163–167

[7] Koch M, Tanami Z, Salomon R (1995) Improved regeneration of shoots from garlic callus. *Hort Sci* 30: 378-383

[8] Shuto H, Abe T, Yamagata T (1993) *In vitro* propagation of plants from root apexderived calli in Chinese chive (*Allium tuberosum* Rottle) and garlic (*A. sativum L.*). *Japan J Breed* 43: 349–354

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

[10] Barandiaran X, Martin N, Rodriguez-conde MF, di-Pietro A, Martin J (1999) An efficient method for callus culture and shoot regeneration of garlic (*Allium sativum* L.). *Hort Sci* 34: 348–349

[11] Luciani GF, Mary AK, Pellegrini C, Curvetto NR (2006) Effects of explants and growth regulators in garlic callus formation and plant regeneration. *Plant Cell Tiss Org Cult* 87: 139–143

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

[13] Myers JM, Simon PW (1999) Regeneration of garlic callus as affected by clonal variation, plant growth regulators and culture conditions over time. Plant Cell Rep 19: 32–36

[14] Zheng S, Henken B, Sofiari E, Jacobsen E, Krens FA, Kik C (1998) Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa*. *Plant Cell Tiss Org Cult* 53: 99-105

[15] Barandiaran X, Martin N, Rodriguez-Conde MF, Di Pietro A, Martin J (1999) Genetic variability on callus formation and regeneration of garlic (*Allium sativum* L.). *Plant Cell Rep* 18: 434-437

[16] Fereol L, Chovelon V, Causse S, Michaux F, Michaux-Ferriere N, Kahane R (2002) Evidence of a somatic embryogenesis process for plant regeneration in garlic (*Allium sativum* L.). *Plant Cell Rep* 21: 197-203

[17] Sata SJ, Bagatharia SB, Thaker VS (2000) Induction of direct somatic embryogenesis in garlic (*A. sativum* L.). *Method Cell Sci* 22: 299–304

[18] Buitveld J, Creemers-Molenaar J (1994) Induction and characterization of embryogenic callus types for the initiation of suspension cultures of leek (*Allium ampeloprosum* L.). *Plant Sci* 100: 195–202

[19a] Eady CC, Lister CE (1998) A comparison of four selective agents for use with *Allium cepa* L. immature embryos and immature embryo derived callus. *Plant Cell Rep* 18: 117–121

[19b] Eady CC, Suo Y, Butler RC (1998) Somatic embryogenesis and plant regeneration from immature embryo cultures of onion (*Allium cepa* L.). *Plant Cell Rep* 18: 111–116

[20] Fracago F, Echeverrigaray S (2001) Micropropagation of Cunila galioides, a popular medicinal plant of south Brazil. *Plant Cell Tiss Org Cult* 64: 1–4

[21] Chu CC, Wang CC, Sun CS (1975) Establishment of an efficientmedium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci Sin* 18: 659–668

[22] Luciani G, Marinangeli PA, Curvetto NR (2001) Increasing nitrate/ammonium ratio for improvement of garlic micropropagation. *Sci Hort* 87: 11–20

[23] Gamborg O, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50: 151–158.