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MICROPROPAGATION OF *ALLIUM WALLICHII* KUNTH, A THREATENED MEDICINAL PLANT OF NEPAL†

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Summary

Bulbs and aerial parts of the Nepalese plant *Allium wallichii* are widely used for medicinal purposes and as a spice. Due to overharvesting the natural populations of the species have been increasingly reduced and the domestication of the species should be considered. For the purpose of the production of plantlets suitable for field culture, a micropropagation procedure based on multiple shoot culture has been established. Multiplication factors of 4.6 on average were possible on MS medium supplemented with 20 μ*M* zeatin. After rooting on MS medium with 10 μ*M* indolebutyric acid, plantlets were acclimatized to greenhouse conditions and transferred to the field with good success.

Key words: Allium wallichii; Alliaceae; domestication; multiple shoot; zeatin.

Introduction

Allium wallichii Kunth (Alliaceae) is an erect herb of 30–40 cm height with slightly thickened and clustered bulbs. It is a perennial plant with a restricted distribution in higher altitudes ranging from 2000 to 4000 m above the sea level in Nepal (Malla, 1976). The bulbous rhizomes are used for the treatment of coughs and colds and against altitude sickness. The young leaves are cooked as a vegetable and dried ones are used as a spice (Manandhar, 1980; Dobremez, 1982). *A. wallichii* is a common ingredient in stomach tonics. The bulb is also boiled, fried with ghee (clarified butter) and used for the treatment of cholera and diarrhea (Coburn, 1984). In the Ayurvedic medicinal system, the indications are tuberculosis, nerve defects, blood circulatory defects, and long life and rejuvenation (Bajracharya, 1979).

Very high amounts of *A. wallichii* are collected from wild habitats, leading to a depletion of the plant resources. The natural multiplication is hindered because entire plants are removed, and in most cases this is done prior to seed setting. It is highly advisable to produce the necessary amounts in field culture for the conservation of the species in its natural environment. It has been noted that micropropagation would be of advantage in the process of the domestication of *A. wallichii* (Malla, 1994).

The application of tissue culture techniques has been described for various species of the genus *Allium*, for example garlic (Novak et al., 1986), onion (Dunstan and Short, 1977; Rauber and Grunewaldt, 1988), chive (Rauber and Grunewaldt, 1988) or *A. carinatum* (Havel and Novak, 1988). No reports on the *in vitro* propagation of *A. wallichii* have been published so far. In the present study we describe a multiplication protocol based on shoot cultures of *A. wallichii*.

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Materials and Methods

Plant material. Seeds of *Allium wallichii* were collected in Godavari, Chandragiri and Langtang (Nepal). After washing under running tap water they were agitated in 50% ethanol for 30 s. They were then treated for 15 min with an aqueous solution of sodium hypochlorite (approx. 2% active chlorine) with a few drops of Tween 20®. After three or four rinses with sterile distilled water, they were aseptically germinated. Four to 6-wk-old seedlings were multiplied to produce secondary explants which in turn were used for the main experiments.

Culture media and culture conditions. Half-strength MS (Murashige and Skoog, 1962) medium was used for seed germination and for rooting, and full-strength MS medium was also used for shoot multiplication and for rooting. The media were supplemented with 3% sucrose (1% for seed germination) and fortified with 0.8% agar (Merck). The pH was set to 5.7 ± 0.1 before autoclaving at 121°C for 20 min. Seed germination, shoot regeneration and rooting were done in glass culture tubes (25 × 150 mm). Baby food jars (60 × 97 mm) were used for routine multiplication and rooting. Rooting and transportation of plantlets was performed in disposable plastic containers (11 × 7.5 × 6 cm). The cultures were kept at 20 ± 1°C and a 16-h photoperiod of 20 μmol m^{-2} s^{-1} cool white fluorescent light (Osram Biolux® tubes).

Shoot multiplication. Seedlings without the root portion were used as primary explants. They were multiplied on basal MS medium supplemented with 10 μ*M* N^6-benzyladenine (BA). Shoots produced on the medium were further multiplied on the same medium to produce a stock. Prior to inoculation on MS media with factorial combinations of BA, kinetin (KN) or zeatin (Z) with indoleacetic acid (IAA), indolebutyric acid (IBA) or naphthaleneacetic acid (NAA), these secondary explants were kept for a few weeks on the medium without growth regulators.

Rooting. Individual shoots (2–3 cm in length) obtained from multiple shoot cultures were inoculated on full- and half-strength MS media supplemented with IAA or IBA in varying concentrations. The cultures were kept at 20 ± 1°C and a 16-h photoperiod of 20 μmol m^{-2} s^{-1} cool white fluorescent light (Osram Biolux® tubes).

Acclimatization and ex vitro *culture.* Plantlets obtained from the *in vitro*-rooting phase were carefully washed with water to remove the agar. Acclimatization and *ex vitro* culture were carried out in Austria (Institute of Pharmacognosy, University of Vienna) and in Nepal (Royal Botanical Garden, Godavari). In Vienna the plantlets were transferred to a gardening soil mixture (Humus-Ton Substrat N8®, Neuhaus, Germany) with 15% perlite (Agroperl®) soaked with an aqueous solution of the fungicides Benlate® (0.05%) and Previcur® (0.1%) and hardened in a mist chamber for

2 wk. In Godavari a mixture of soil and sand (1:1) in plastic boxes with transparent covers was used for acclimatization. In both cases the plants were kept at high humidity for 1 wk, then the humidity was reduced to greenhouse level within the second week. After a further 1–2 wk in the greenhouse they were then transferred to test plots.

Statistical analysis. Each experiment was performed twice using 18 explants. The results were recorded after either 4 (rooting) or 6 wk (multiplication experiments). Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze the data. These tests were done using the Statistica software package by StatSoft, Inc.

Results and Discussion

Culture establishment and production of secondary explants. The initiation of axenic cultures did not pose major problems. Surface-sterilized seeds were 95% free of contaminants and germinated in a few days. Due to the limited number of available seeds a first multiplication step was included to produce secondary explants for the main experiments. For this purpose the root portion of 6-wk-old seedlings was removed and we inoculated the primary explants on MS medium supplemented with 10 μ*M* BA. An average multiplication factor between three and four (data not shown) was obtained after a 6-wk culture. One vigorous clone was selected for the further investigations: the shoots were separated and repeatedly transferred to the same medium. This procedure finally resulted in a large number of shoots which were subsequently used as secondary explants for the main experiments. Prior to use they were always kept for a few weeks on the half-strength MS medium without growth regulators.

Adventitious shoot formation. To increase the multiplication factor, either whole shoots or longitudinally split shoot halves (LH) were inoculated on the full-strength MS media supplemented with 108 factorial combinations of the cytokinins BA, KN and Z (0, 5, 10, or 20 μ*M*) and the auxins IAA, IBA and NAA (0, 1 or 5 μ*M*). Whole explants always showed less response than LH inocula regardless of the culture media (Tables 1 and 2). This coincided with the results in *Narcissus* sp. (Squires and Langton, 1990), *Yucca glauca* (Bentz et al., 1988), or *Opuntia polyacantha* (Mauseth and Halperin, 1975). When compared to medium with 10 μ*M* BA (three to four shoots) enhanced shoot formation was observed when the concentration of the cytokinin was lowered to 5 μ*M* and 1 μ*M* IAA was added (whole shoot explants). However, an average multiplication factor of 4.6 was obtained when LH explants were inoculated on medium with 20 μ*M* BA and 5 μ*M* IAA. The same rate of shoot formation was also found on media containing Z, as shown for LH explants in Table 1 and for whole shoot explants in Table 2. With LH explants 20 μ*M* Z alone again produced 4.6 shoots on average, but the same factor was also achieved when the level of Z was reduced to 10 μ*M* and combined with 5 μ*M* IBA. Combinations of Z with 1 μ*M* IBA were not favorable for shoot regeneration. This trend was not observed if the cytokinin was combined with IAA or NAA: irrespective of the level of auxin the regeneration factor on medium with 20 μ*M* Z alone was always significantly reduced when either of the auxins were added. Ten μ*M* Z alone was less efficient than a combination with 1 μ*M* IAA, while the addition of 1 or 5 μ*M* NAA did not influence shooting. Shoot regeneration on medium supplemented with 5 μ*M* Z did not differ from that obtained on medium free of growth regulators. However, a combination of 5 μ*M* Z with either 1 μ*M* IAA or NAA, or with 5 μ*M* IBA significally enhanced the formation of shoots.

TABLE 1

EFFECTS OF DIFFERENT CONCENTRATIONS (μ*M*) OF ZEATIN COMBINED WITH AUXINS ON SHOOT REGENERATION FROM LONGITUDINALLY SPLIT SHOOT EXPLANTS OF *ALLIUM WALLICHII* AFTER 6 WK OF CULTURE

Zeatin	IAA	Mean ± SE*	IBA	Mean ± SE*	NAA	Mean ± SE*
0	0	2.00 ± 0.07 a	0	2.00 ± 0.11 bc	0	2.00 ± 0.10 a
0	1	2.31 ± 0.25 ab	1	2.16 ± 0.23 bc	1	1.91 ± 0.15 a
0	5	2.18 ± 0.13 ab	5	1.33 ± 0.07 a	5	1.50 ± 0.08 a
5	0	1.75 ± 0.13 a	0	1.83 ± 0.12 ab	0	1.75 ± 0.13 a
5	1	2.75 ± 0.20 bc	1	2.05 ± 0.17 bc	1	2.93 ± 0.23 b
5	5	2.94 ± 0.24 c	5	3.27 ± 0.23 d	5	1.87 ± 0.14 a
10	0	3.25 ± 0.24 c	0	3.11 ± 0.18 d	0	3.25 ± 0.19 b
10	1	3.87 ± 0.24 d	1	2.05 ± 0.17 bc	1	3.18 ± 0.11 b
10	5	3.37 ± 0.24 cd	5	4.65 ± 0.20 e	5	2.68 ± 0.17 b
20	0	4.69 ± 0.25 e	0	4.68 ± 0.27 e	0	4.68 ± 0.27 c
20	1	3.00 ± 0.17 c	1	2.50 ± 0.15 c	1	2.87 ± 0.18 b
20	5	3.00 ± 0.19 c	5	4.66 ± 0.27 e	5	3.16 ± 0.25 b

Values are mean ± SE of two replicates with 18 explants.

Mean values with the same letters are not significantly different at the 5% level (Duncan's multiple range test).

TABLE 2

EFFECTS OF DIFFERENT CONCENTRATIONS (μ*M*) OF ZEATIN COMBINED WITH AUXINS ON SHOOT REGENERATION FROM WHOLE SHOOT EXPLANTS OF *ALLIUM WALLICHII* AFTER 6 WK OF CULTURE

Zeatin	IAA	Mean ± SE	IBA	Mean ± SE	NAA	Mean ± SE
0	0	2.33 ± 0.12 bcd	0	2.34 ± 0.14 de	0	2.50 ± 0.13 cde
0	1	1.87 ± 0.24 ab	1	1.88 ± 0.12 bcd	1	1.69 ± 0.12 ab
0	5	1.93 ± 0.13 ab	5	1.38 ± 0.09 a	5	1.50 ± 0.08 a
5	0	2.06 ± 0.09 abc	0	1.94 ± 0.19 cd	0	2.05 ± 0.14 bc
5	1	1.94 ± 0.16 ab	1	1.44 ± 0.12 ab	1	2.87 ± 0.19 e
5	5	1.80 ± 0.18 a	5	2.05 ± 0.16 cde	5	2.25 ± 0.13 cd
10	0	2.45 ± 0.20 cde	0	2.47 ± 0.18 e	0	2.47 ± 0.17 cde
10	1	2.37 ± 0.15 bcd	1	1.41 ± 0.16 ab	1	2.68 ± 0.15 de
10	5	2.93 ± 0.20 e	5	3.88 ± 0.21 g	5	2.50 ± 0.17 cde
20	0	3.44 ± 0.12 f	0	3.41 ± 0.27 f	0	3.42 ± 0.15 f
20	1	2.75 ± 0.14 de	1	1.77 ± 0.19 abc	1	2.81 ± 0.18 e
20	5	2.56 ± 0.14 cde	5	3.11 ± 0.20 f	5	2.81 ± 0.13 e

Values are means ± SE of two replicates with 18 explants.

Mean values with the same letters are not significantly different at the 5% level (Duncan's multiple range test).

None of the media supplemented with BA or KN, alone or combined with the auxins, led to significantly different effects than the Z-containing formulations except of a combination of 20 μ*M* BA with 5 μ*M* IAA. These findings were in contrast to similar investigations with other *Allium* species. For example, *A. tuberosum* was successfully multiplied on MS medium supplemented with 0.5 mg l^{-1} BA (Pandey et al., 1992). BA alone (4.4 μ*M*) was also favorable for multiple shoot cultures of *A. ascalonicum*, while 8 μ*M* BA combined with 0.1 μ*M* NAA was successfully used for the *in vitro* propagation of garlic (Mohamed-Yasseen et al., 1994).

Rooting. Only a limited number of micropropagated shoots formed a few short adventitious roots when cultured on either full- or half-strength MS medium without growth regulators (Table 3). When the auxins IAA and IBA were added in concentrations of 1, 5,

TABLE 3

EFFECT OF AUXINS ON ROOTING OF *IN VITRO*-FORMED SHOOTS OF *ALLIUM WALLICHII* IN FULL- AND HALF-STRENGTH MS MEDIA AFTER 4 WK OF CULTURE

		Full MS			Half MS		
Auxins	μ*M*	Rooting (%)	No. of roots	Root length (mm)	Rooting (%)	No. of roots	Root length (mm)
Control	0	30.0	0.3 ± 0.1	3.0 ± 2.1	31.2	0.41 ± 0.1	3.1 ± 0.8
IAA	1	100	1.72 ± 0.1	7.2 ± 1.3	100	2.12 ± 0.1	8.7 ± 0.8
	5	94.4	2.66 ± 0.3	9.7 ± 0.8	100	2.29 ± 0.1	9.4 ± 0.6
	10	77.8	1.55 ± 0.2	10.0 ± 1.6	94.1	1.65 ± 0.1	11.2 ± 1.4
IBA	1	77.7	1.22 ± 0.3	7.8 ± 1.5	100	2.18 ± 0.2	12.5 ± 1.3
	5	76.3	1.55 ± 0.3	7.7 ± 1.4	94.3	2.05 ± 0.1	10.5 ± 0.5
	10	100	2.22 ± 0.1	12.2 ± 1.5	100	1.94 ± 0.1	13.3 ± 0.1

Values are means ± SE of two replicates with 18 explants.

or 10 μ*M*, most of the media were effective for rooting. Plantlets produced on any of these media could be transferred *ex vitro* without losses.

Acclimatization and ex vitro *culture.* The main experimental work has been conducted at the Institute of Pharmacognosy in Vienna. At this location an automated mist chamber was available where the relative humidity could be exactly controlled and regulated. Acclimatization of *A. wallichii* plantlets was easy when the relative humidity was lowered from initially 75% to 50% in 2 wk. Subsequently, the plants were moved to the greenhouse where they exhibited vigorous growth. A few plants were also transferred to the garden of the institute and they set flowers after a few weeks. To test the performance of *in vitro*-derived plants under the specific conditions in Nepal, a set of 300 shoots were rooted on MS medium with 10 μ*M* IBA using disposable plastic containers. These containers were obviously better suited for transportation than glass containers. In Godavari (Nepal) the infrastructure for acclimatization was different. First, no automated humidity regulation was possible in the local greenhouse. Therefore we used plastic boxes with transparent covers which were gradually opened, again for a total of 14 d. Also, the substrate for cultivation was different: we used a mixture of plain soil and sand (1:1) instead of a gardening mixture with a high content of humus and peat. No fungicides were available in Nepal either. Nevertheless, all 300 plantlets could be successfully conditioned to the greenhouse. Subsequently, 100 plants were transferred to an experimental field plot in April 1999. While initially vigorous growth was observed, with the beginning of the monsoon rain in May some 60% of the plants died. However, the remaining specimens developed normally and did not show any morphological aberrations.

In conclusion, we may state that *Allium wallichii* can be propagated *in vitro* with sufficient multiplication factors to allow the production of plantlets for further *ex vitro* cultivation. It will, however, be necessary to carry out further research locally in Nepal, especially concerning the parameters for field culture such as suitable time of the year for transfer outdoors, proper soil composition, and need for fertilizer. Our results indicate that the application of tissue culture in the process of domestication of *A. wallichii* may contribute to the protection of the natural populations in Nepal.

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