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Micropropagation of *Asparagus cooperi* as affected by growth regulators

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Abstract

For clonal propagation of *Asparagus cooperi*, shoot tips and node explants of 7, 20 and 35 d old spear from the region within 10 cm and below 25 cm from the apex were cultured in modified Murashige and Skoog's (1962) medium containing 6-benzylaminopurine (BAP). The required concentration of BAP varied in explants of different ages and types. In shoot tip culture, the rate of shoot multiplication was higher after 40 d than 60 d of culture. The maximum number (62 - 65) of shoots were obtained from shoot tip explants of 20 d old spear in the medium containing 2.0 mg dm^{-3} of BAP, 80 mg dm^{-3} of adenine and 0.02 mg dm^{-3} of α-naphthalene acetic acid after 60 d of culture. From node cultures, high number of shoots were obtained after 30 d. Pretreatment with BAP in liquid medium for 48 h was effective for semire-juvenescence. The individual shoots produced roots in presence of indole-3-butyric acid and also in potassium salt of indole-3-butyric acid, the later being more effective. All regenerants were cytologically stable.

Introduction

Micropropagation is an useful alternative to the conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication (Hussey 1986, Murashige 1990). Shoot proliferation is usually employed as it provides genetic stability. This technique generally avoids chimera formation, prevents somaclonal variation and yields a large number of uniform regenerated plants. The genus *Asparagus* comprising more than 300 species is an important member of monocotyledons in which shoot culture has so far been reported only in *A. officinalis* (Reuther 1984).

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; KIBA - potassium salt of indole-3-butyric acid; NAA - α-naphthalene acetic acid; Ade - adenine; 2ip - N_6-(2-isopentenyl)-adenine; Kin - kinetin; MS medium - medium of Murashige and Skoog (1962).

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The species of *A. cooperi* is valued for its horticultural (Ghosh and Sen 1991, 1992) as well as medicinal importance because of sapogenin content (Kar and Sen 1986). In this species, the propagation through somatic embryogenesis and organogenesis has been reported by the present authors (Ghosh and Sen 1989, 1991, 1992). The present paper deals with the effectiveness of shoot and node culture as a means of mass propagation controlled by different factors and with the analysis of chromosome constitution of regenerated plants of *A. cooperi*.

Materials and methods

The species *Asparagus cooperi* Baker. grows in the eastern Himalayan region. Plants were collected from Chandra Nursery, Sikkim and grown at the experimental garden, Department of Botany, University of Calcutta. Two different types of explants namely shoot tip (1 cm ± 2 mm) and node (0.5 cm) were used. These explants were collected from apical region (within 10 cm from the apex) and basal region (below 25 cm downwards from the apex) of spears of 7 d (10 - 12 cm total length), 20 d (35 - 45 cm total length) and 35 d (60 - 70 cm total length) old plants. Explants of basal region from 7 d old spear could not be collected due to short length of spear. The explants were sterilized by washing with 5 % liquid detergent *Teepol* followed by gently shaking in 0.1 % mercuric chloride for 6 - 8 min and finally rinsing five times in sterile redistilled water. For culturing, MS medium (macro- and micro-salts) was used supplemented with 2050 mg dm^{-3} NH_4NO_3, 250 mg dm^{-3} KH_2PO_4, 150 mg dm^{-3} myo-inositol, 4 mg dm^{-3} thiamine, 3 mg dm^{-3} glycine, 400 mg dm^{-3} L-arginine, 3 % saccharose and for solidification 0.7 % agar was added. For shoot multiplication different cytokinins like BAP, Kin, Ade, 2ip (0.5, 1.0, 2.0, 3.5, 5.0, 7.0, 10 mg dm^{-3}) alone or in combinations were applied. For rooting, MS-R medium was used containing half-strength MS macro-salt, full strength micro-salt, 3 mg dm^{-3} riboflavin, and 2 % sucrose. The auxins IAA, NAA, IBA, KIBA were also added.

The pH of the media was adjusted to 5.7 before autoclaving. The media were sterilized by autoclaving for 15 min at 121°C and 1.03 kg cm^{-2} pressure. Cultures were kept under 16/8 h light/dark cycle, irradiance of 42.25 µmol m^{-2} s^{-1}, temperature of 25 ± 1 °C and relative humidity of 55 %.

Results

Shoot explants from apical region: Multiplication of shoots was induced by BAP, Ade and NAA. Explants from 7 d old spear responded well in the medium containing 1 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA. Within 12 d 6 - 8 shoots were produced. After subculturing, 38 - 43 shoots developed after 40 d and after 60 d the number of shoots increased to 46 - 50. The explants of 20 d old spear produced 12 - 14 shoots after 12 d in the medium containing 2.0 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA. After subculturing, 45 - 48 shoots were produced after 40 d and 62 - 65 shoots (Fig. 1) in 60 d. The explant from 35 d old spear produced

8 - 10 shoots after 12 d in the medium containing 3.0 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA and after subculturing developed 28 - 25 shoots after 40 d and 42 - 47 shoots after 60 d (Fig. 5).

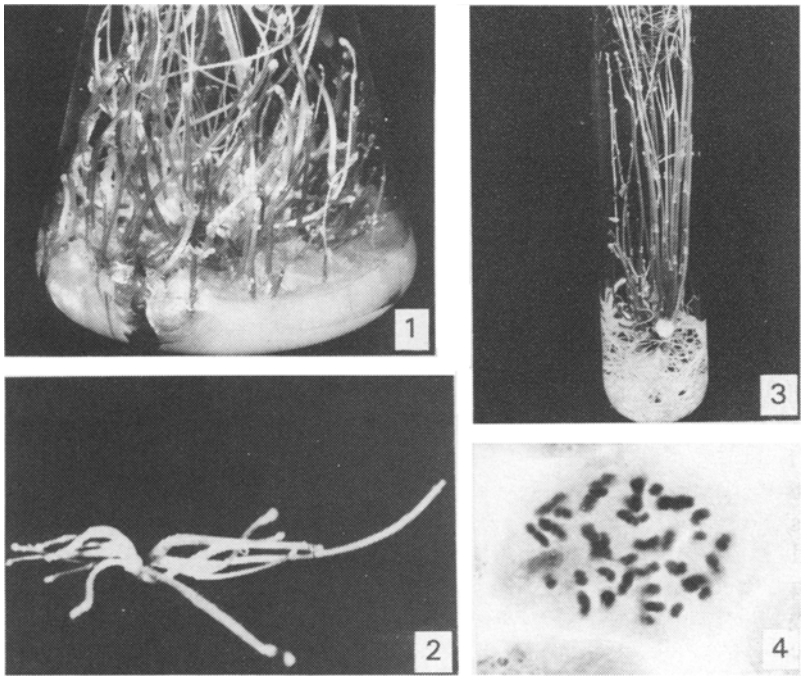
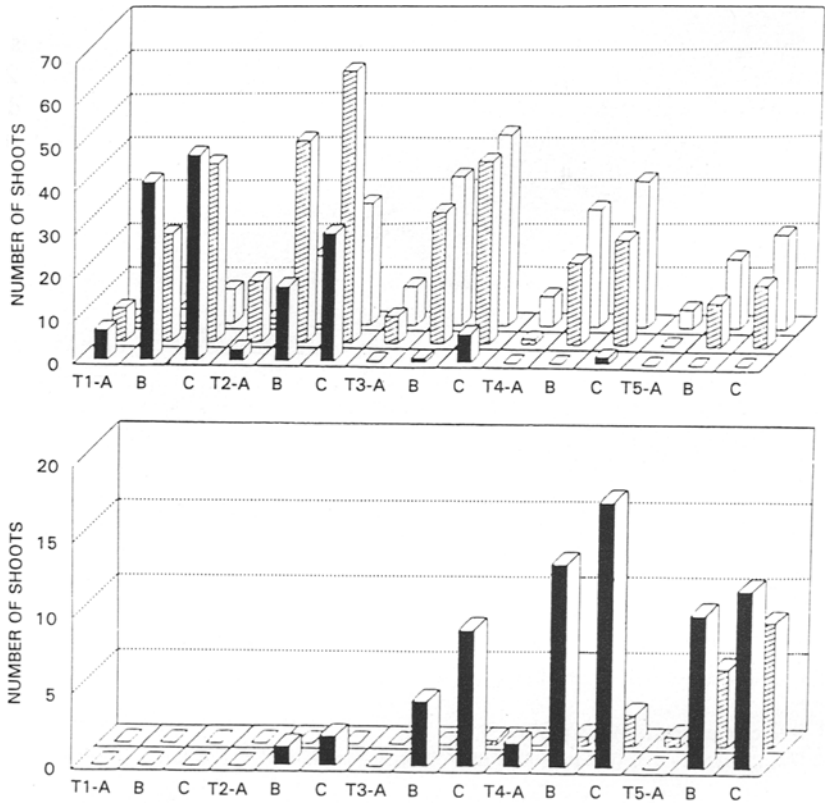


Fig. 1. Shoot multiplication of *A. cooperi* through shoot subculture for 60 d.
Fig. 2. Multiple shoot formation during 30 d culture of nodal explant from 20 d old spear of *A. cooperi*.
Fig. 3. Rooted plantlets of *A. cooperi*.
Fig. 4. Metaphase plate of root tip cell of micropropagated plant of *A. cooperi* (2n = 40).

Shoot explants from basal region: Explants of 20 d old spear cultured in the medium containing 2.0 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA produced 1 - 2 shoots after 40 d. The increase in the concentration of BAP to 3.5 mg dm^{-3}, Ade to 80 mg dm^{-3} in the presence of 0.02 mg dm^{-3} NAA promoted shoot multiplication and after 40 d 12 - 14 shoots were produced (Fig. 6). The induction of shoot could be increased to 31 - 34 in the same medium used for apical region when the explants were pretreated for 48 h in medium containing 4.0 mg dm^{-3} BAP in a shaker. Such pretreatment was also fruitful for the explants of 35 d old spear producing 21 - 24 shoots in 40 d. In the absence of such pretreatment only 4 - 5 shoots were induced after 40 d even in presence of 5 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA. Elongation of shoots was promoted by addition of 0.5 mg dm^{-3} 2ip.

Node explant from apical region: The node explant of apical region of 7 d old spear produced 6 - 8 axillary shoots after 30 d when cultured in the basal medium

containing 1.5 mg dm^{-3} BAP and 0.02 mg dm^{-3} NAA. The explant from 20 d old spear showed optimum multiplication of 12 - 15 axillary shoots (Fig. 2) in 30 d in the medium containing 2.5 mg dm^{-3} BAP and 0.02 mg dm^{-3} NAA. Nearly 7 - 10 axillary shoots were produced in 3.0 mg dm^{-3} BAP and 0.02 mg dm^{-3} NAA after 30 d from the node of 35 d old spear (Fig. 7).



Figs. 5 and 6. Effect of different concentrations of BAP (in the presence of 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA), age of spear (7 d - *closed columns*, 20 d - *hatched columns* and 35 d - *open columns*) and age of culture on multiplication of shoots from shoot explants of apical region (Fig. 5) or of basal region (Fig. 6). T1 - 1 mg dm^{-3} BAP, T2 - 2 mg dm^{-3} BAP, T3 - 3 mg dm^{-3} BAP, T4 - 3.5 mg dm^{-3} BAP, T5 - 5 mg dm^{-3} BAP. A - 12 d, B - 40 d and C - 60 d of culture].

Node explant from basal region: The explants of 20 d old spear did not respond when cultured in the same basal medium used for apical region. But increasing the concentration of BAP from 2.5 to 3.5 mg dm^{-3} in the presence of 0.02 mg dm^{-3} NAA induced 1 - 2 axillary shoots after 30 d of culture. In explants of 35 d spear, 1 - 2 axillary shoots could be induced after 50 d by increasing the concentration of BAP to 6 mg dm^{-3} (Fig. 8).

The number of shoots formed was increased by keeping the explants in the liquid basal medium containing 5 mg dm^{-3} BAP in a shaker for 48 h and then transferring to

the agar medium supplemented with 1.5 mg dm^{-3} BAP, 50 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA. In this case after 30 d 6 - 7 axillary shoot buds were produced from node explant of 20 d old spear. From the explants of 35 d old spear, 2.0 mg dm^{-3} BAP, 80 mg dm^{-3} Ade and 0.02 mg dm^{-3} NAA was suitable for formation of 4 - 6 axillary shoots. For elongation of shoot 0.5 mg dm^{-3} 2ip was added. Secondary shoots could not be obtained from node culture.

Induction of root in excised shoots: Initiation of roots from the growing shoots (2.0 - 2.5 cm) was successful when transferred to MS-R basal medium containing 2.0 mg dm^{-3} of IBA (67 - 70 % of shoots produced roots during 11 - 14 d) or 2.0 mg dm^{-3} of KIBA (83 - 87 % of shoots produced roots within 7 - 10 d) (Fig. 3). Dark condition was more suitable for rooting (Table 1).

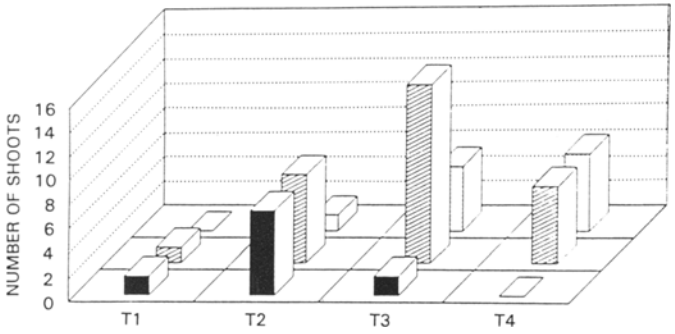


Fig. 7. Effect of different concentrations of BAP (T1 - 0.5 mg dm^{-3}, T2 - 1.5 mg dm^{-3}, T3 - 2.5 mg dm^{-3}, T4 - 3.5 mg dm^{-3}) in the presence of 0.02 mg dm^{-3} NAA on number of axillary shoots produced during 30 d on node explant from apical region (age of spear 7 d - *closed columns*, 20 d - *hatched columns*, 35 d - *open columns*).

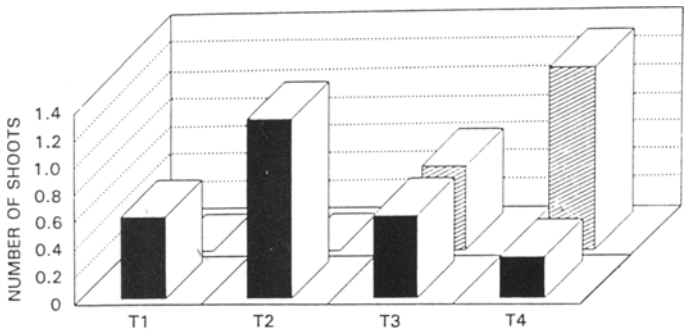


Fig. 8. Effect of different concentrations of BAP (T1 - 3.0 mg dm^{-3}, T2 - 3.5 mg dm^{-3}, T3 - 5.0 mg dm^{-3}, T4 - 6.0 mg dm^{-3}) in the presence of 0.02 mg dm^{-3} NAA on number of axillary shoots produced on node explant from basal region (age of spear 20 d, cultivation 30 d - *closed columns*, age of spear 35 d, cultivation 50 d - *hatched columns*).

Table 1. Effect of IBA and KIBA on the rooting of *A. cooperi* excised shoots in a 16/8 h light/dark cycle and in complete dark culture (50 shoots per treatment, mean of 3 replication ± S.D.).

Growth regulator [mg dm^{-3}]		Light/dark cycle percentage of rooting	number of roots	rooting time [d]	Dark percentage of rooting	number of roots	rooting time [d]
IBA	(0.5)	36.6 ± 2.30	1.6 ± 1.54	16-20	48.0 ± 1.73	2.3 ± 1.15	15-17
IBA	(1.0)	44.3 ± 1.15	2.3 ± 2.08	16-19	61.6 ± 2.51	3.0 ± 1.73	12-15
IBA	(2.0)	54.6 ± 1.54	2.6 ± 1.73	13-15	69.6 ± 2.51	3.3 ± 1.52	11-14
IBA	(3.0)	46.3 ± 1.52	2.6 ± 1.73	15-17	61.0 ± 1.73	2.6 ± 2.08	13-16
KIBA	(0.5)	46.6 ± 2.08	1.6 ± 1.15	14-16	59.6 ± 2.08	2.6 ± 0.57	9-12
KIBA	(1.0)	62.0 ± 1.73	3.0 ± 2.64	11-14	76.6 ± 2.08	4.0 ± 1.00	9-11
KIBA	(2.0)	73.3 ± 1.15	3.3 ± 1.73	10-12	86.6 ± 1.52	5.3 ± 0.57	7-10
KIBA	(3.0)	65.6 ± 1.15	2.0 ± 1.20	11-13	73.3 ± 1.52	3.0 ± 1.73	10-11

Cytological study: Ninety regenerants were analysed from two explants sources. Of these, 30 plants each from different sets were cytologically analysed from root tip tissues. Twenty five metaphase plates from each root tip were examined. All the cells showed 2n = 40 chromosomes (Fig. 4) like that of the source plant. Structural dissimilarity could not be located, too.

Discussion

The multiplication of shoots is one of the most important features of *in vitro* propagation. The major factor influencing multiplication rate is the interaction of the physiological state of the plant material with the culture medium. Propagation rate of different explants may also vary to a great extent (Chen and Evans 1990). In the culture medium for shoots multiplication cytokinins are still indispensable and BAP often form essential component in the medium (Hu and Wang 1983) which is also noted in the present study. For multiplication of shoots, the required concentration of BAP varied in different explant sources. In general, with the increased age of the explant there was the need for higher concentration of BAP for shoot multiplication. Similarly, the region below 25 cm from the apex did not respond in a similar way as that of the apical region. The differential requirements may be attributed to the varying levels of endogenous cytokinin in explants from plants of different age or from different position on plants.

In relation to period of culturing, 60 d old culture yielded better multiplication than 40 d old one. But the potential for further multiplication was conserved only up to 40 d. In the older culture, that is 60-d or more, there was no further multiplication of shoots even though the number of shoots initially developed were high. Evidently the age of culture in this species is one of vital importance to preserve the potential for shoot multiplication for a long period.

The application of BAP also influenced the rate of multiplication. High concentration of BAP following prolonged incubation lead to the production of

average number of shoots. But if it is pretreated with BAP in a shaker for 48 h, rejuvenescence of the mature explants is triggered effectively. The importance of BAP as antiageing agent has also been reported (Pierik 1990).

It may, therefore, be concentration of BAP (A), position of explant (B), age of spear (C) and culture period (D) interact for securing mass multiplication and prolonged viability. The four way of analysis of variance (ANOVA) indicates a highly significant ($P = 0.1$) interaction (A × B × C × D) among these factors. Any one character can influence shoot multiplication.

The absence of secondary shoot formation from the nodal explant is rather of special interest. It is unlike that of shoot tip explant, there is profuse growth of secondary shoots. It is likely, that endogenous hormones needed for shoot formation are present in the shoot apex and it is translocated to the site of secondary shoot growth. In the nodal region its absence may be responsible for lack of growth of secondary shoots. The analysis of variance reveals that the age of spear (A), position of explant (B) and medium (C) interaction (A × B × C) is highly significant.

For rooting, it was noted that KIBA was more effective than IBA. It is likely, that application of KIBA might increase the level of K^+ and accelerate rooting.

All the regenerants so far analysed do not show any visible cytological changes. The chromosome number ($2n = 40$) remains the same without any marked structural alterations. Apparently, the regenerants are stable diploid. The data obtained in the present study may be effectively employed to secure apparently stable diploids through *in vitro* propagation.

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