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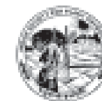

A PROTOCOL FOR *IN VITRO* PROPAGATION OF BER *(ZIZIPHUS JUJUBA)*

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SUMMARY

A protocol for micropropagation of *Ziziphus jujuba* was developed by using nodal explants in different media compositions. Various morphogenic responses of explants have been observed by using different media, *viz.* MS, B_5 and N_6. MS basal medium was found to be the best among them for shoot proliferation (93.75 %). Shoot development was initiated within a week. No callus development was observed. In order to enhance the shoot regenerative potential, explants were cultured on MS medium supplemented with various cytokinins, *viz.* BAP, kinetin and TDZ. MS medium supplemented with BAP (1.78 µM) was found to be ideal for shoot development. Among various auxins used, 2.69 µM NAA proved to be the best to induce healthy roots with minimum callused thickening, resulting in maximum survival of plants (80 %) in the field.

Key words: **Ber, micropropagation, nodal shoots, *Ziziphus jujuba***

INTRODUCTION

Ber or Indian jujuba belongs to the genus *Ziziphus* of the family Rhamnaceae. *Ziziphus jujuba* is a vigorous grower and has a rapidly developing tap root. It is a multipurpose plant with many medicinal properties. Its fruits are used in indigestion, biliousness and applied on cuts. The seeds are taken to halt nausea, vomiting, and abdominal pains in pregnancy. The leaves are helpful in liver troubles, asthma and fever. The powdered root is dusted on wounds. Juice of the bark from roots is said to alleviate gout and rheumatism. Ripe fruits are mostly consumed raw, but are sometimes stewed. Its wood has been used to make agricultural implements, household utensils, toys and general turnery. It is also valued as firewood and it is a good source of charcoal and activated carbon.

Ziziphus is vegetatively propagated through grafting or budding. Micropropagation offers the advantage of rapid multiplication of such multipurpose plants in terms of time and space with no limitation of growth seasons. Attempts have also been made to regenerate plantlets of *Ziziphus* cultivars via callus formation from explants such as nodal segments, leaf, cotyledon, root, and hypocotyl followed by organogenesis and somatic embryogenesis (Cheong and Kim 1984, Kim *et al.* 1987, Mathur *et al.* 1993, Mitrofanova and Shevelukha 1995). In the present investigation, a protocol has been developed for multiplication of *Ziziphus jujuba* through micropropagation by using nodal explants in various media compositions.

MATERIALS AND METHODS

Plant material was collected from Research Farm of R.B.S. College, Khandari, Agra, Uttar Pradesh, India (27.2° N, 77.9° E, 163 m). Nodal segments (1.5 – 2.0 cm long) taken from four year old trees of *Ziziphus jujuba* were used as explants. They were kept under tap water with soap solution (4-5 drops) for 20 minutes and were stirred in water with Tween 20 (surfactant, 4-

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5 drops) for 10 minutes. Explants were surface sterilized by following treatments: *i.* with bavistin (0.5 %, w/v) for 30 minutes, ii. with streptomycin and chloramphenicol (each 0.1 %, w/v) either alone or in combinations for 30 minutes, and iii. with either varying concentrations of $HgCl_2$ (0.1 %, w/v), spirit or in combinations for varying time interval. After each treatment explants were washed thrice with sterilized distilled water. Explants were also subjected to treatment with various combinations of antioxidants (ascorbic acid, citric acid and poly vinyl pyrrolidon or PVP, 0.1 – 1.0 %) into horizontal shaker for 15 - 30 minutes to check the release of phenol compounds from explant.

Three types of tissue culture media were used for raising cultures. These were MS (Murashige & Skoog 1962), Gamborg's B5 medium (B_5) (Gamborg *et al.* 1968) and Nitsch or N_6 (Nitsch & Nitsch 1969). The MS culture medium chiefly consisted of inorganic nutrients, vitamins: thiamine-HCl (5 mg l^{-1}), pyridoxin-HCl (12.5 mg l^{-1}) and nicotinic acid (12.5 mg l^{-1}); sucrose (3 %, w/v), agar agar (0.8 %, w/v) and antioxidants: ascorbic acid, citric acid and PVP. Adenine sulphate (100-200 mg l^{-1}), L-glutamine (100-200 mg l^{-1}) and glycine (50 mg l^{-1}) were also added to the medium. For shoot differentiation, MS medium was supplemented with varying concentrations of cytokinins, 6-benzylaminopurine (BAP, 0.88-3.55 µM), kinetin (Kn, 0.93-3.72 µM) or thidiazuran (TDZ, 0.91-3.63 µM). Varying concentrations of some antioxidants - ascorbic acid (50 mg l^{-1}), citric acid (50 mg l^{-1}) and PVP (100 mg l^{-1}) were used with MS medium to check the release of phenols into medium from explants. All media were adjusted to pH 5.8 ± 0.2 before adding agar agar and sterilized by autoclaving at 15 lbs pressure at 121°C for 16 minutes. For rooting, MS media (+ 1.78 µM BAP) with different concentrations of various auxins, *viz.* 2.85-5.71µM indole acetic acid (IAA), 2.46-4.90 µM indole butyric acid (IBA) or 2.69-5.37 µM naphthalene acetic acid (NAA).

Cultures were incubated at 25 ± 2 °C under 16 hours light and 8 hours dark cycle with irradiance by cool fluorescent tubes. The explants were regularly transferred into new tubes with fresh media to check the browning of explants. The experiment was repeated three times and the reported data are mean of the three experiments.

RESULT AND DISCUSSION

Nodal explants proved as good explants as they remained fresh and green even after treating with $HgCl_2$. But, there were two problems with nodal explants, first the inherent contamination in the explants and second, the problem of phenolic leaching. Sterilization of explants with 0.1 % $HgCl_2$ (for 3 minutes) after treating with fungicide bavistin and antibiotics (streptomycin and chloramphenicol) was very effective to control upto 100 % contamination with good survival rate (75-90 %). However, if the concentration and duration of $HgCl_2$ was increased, the explants turned brown and ultimately died. Use of antioxidants, liquid medium and the incubation of cultures in the dark were the prerequisites for the removal of browning of explants as well of the medium due to the presence of the phenols in the nodal explants. These phenols after oxidation changes into quinones, which are inhibitory to plant growth (Mishra 2004). Therefore, the nodal segments were sub cultured into fresh medium after every one or two day depending on the browning of the medium. Introduction of antioxidants into medium did not reduce the browning of explants significantly but retransfer into new tube with fresh medium reduces browning of explants effectively, it also increased the rate of survival of explants (80 %).

Various morphogenic responses of explants have been observed by using different media, *viz.* MS, G_5 and N_6 basal media with various compositions (Table 1). MS basal medium proved to be the best among them for shoot proliferation (93.75 %). Shoot development was initiated within a week at 25±2°C under 16 hours light and 8 hours dark cycle (Fig. 1a). No callus development was noted. Among various concentrations of cytokinins, 1.78 µM of BAP proved to be most suitable (Table 2)

Table 1. Morphogenic response of *Ziziphus jujuba* nodal explants, reared on different basal media after 30 days of incubation

Medium	Explants forming shoots (%)
MS	93.75
B5	79.16
N_6	83.33

Table 2. Effects of BAP, kn and TDZ on axillary shoot proliferation in *Ziziphus jujuba* after 30 days of incubation in semisolid MS medium

BAP (µM)	Explants forming shoots (%)	Kinetin (µM)	Explants forming shoots (%)	TDZ (µM)	Explants forming shoots (%)
0	75	0	67	0	71
0.88	83	0.93	79	0.91	75
1.78	92	1.86	83	1.82	83
2.66	83	2.79	83	2.72	88
3.55	83	3.72	67	3.63	79

for obtaining maximum number of axillary shoots after 20-45 days of culture incubation in the fresh medium (Fig. 1b-c). BAP has earlier been reported the most active among cytokinins (Bonga 1992).

A good percentage of rooting (upto 83.3 %) could be achieved in various concentrations of the auxins, like IAA, IBA and NAA used together with 1.78 µM BAP (Table 3) but all plants could not survive during hardening. It is the length, number, colour, thickness and quality of the root, which was responsible for the survival of the plants in the field. The results of rooting (Fig. 1d) as affected by different auxins had been presented in Table 3. Among various auxins used, 2.69 µM NAA (with 1.78

Table 3. Effect of various auxins IAA, IBA and NAA supplemented to MS + 1.78 µM BAP on rooting response of *in vitro* shoots of *Ziziphus jujuba*

Auxin (µM)	Explant forming roots (%)	Rooting response
2.85 IAA	79.16	Callused, brown
5.71 IAA	45.83	Callused, white
2.46 IBA	62.50	Callused, brown
4.90 IBA	66.67	Callused, white
2.69 NAA	83.33	Callused, white
5.37 NAA	70.83	Callused, white

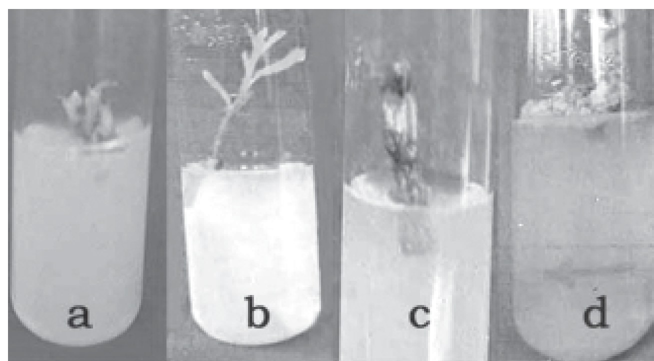

Fig. 1 (a-d): *In-vitro* micropropagation of *Ziziphus jujuba:* a. Developing *in-vitro* shoot after 7 days on basal MS medium, b. Developed *in-vitro* shoot after 20 days on MS + 1.78 µM BAP, c. Developed *in-vitro* shoot after 45 days on MS + 1.78 µM BAP, d. Developed callus after 60 days on MS + 1.78 µM BAP + 2.69 µM NAA

µM BAP) proved to be the best to induce healthy roots with minimum callused thickening, resulting in maximum survival of plants (80 %) in the field. In present investigation, it has been observed that callus induction was also seen just after root initiation and this callusing stopped when they were transferred immediately after root initiation to fresh medium without any hormone. Similar results were seen by Sudhersan and Hussain (1993). They cultured nodal segments and shoot tips of *Ziziphus spina christi* and found 30 % rooting in the medium containing 10 mg/litre IBA and callus induction was also seen just after root initiation. Rooted plantlets were transferred to plastic cups containing sterilized vermiculite for hardening. Transplanted young plantlets in vermiculite in pots were acclimatized and hardened successfully with in two weeks.

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