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ORIGINAL ARTICLE

Efficient *in vitro* direct shoot organogenesis from seedling derived split node explants of maize (*Zea mays* L.)

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Abstract Maize is one of the important cereal crops around the world. An efficient and reproducible regeneration protocol *via* direct organogenesis has been established using split nodes as ex-plants derived from 7 to 10 day old *in vitro* grown seedlings. Surface sterilized maize seeds were germinated on MS medium lacking plant growth regulators. Nodal sections of 7–10 day old seedlings were isolated, split longitudinally into two halves and cultured on regeneration medium containing different concentrations of 6-benzyladenine (2.20, 4.40, 6.60, 8.80, 11.0 and 13.2 μM) or kinetin (2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM). Inclusion of 8.80 μM BA into MS supplemented medium triggered a high frequency of regeneration response from split node explants with a maximum number of shoots (12.0 ± 1.15) and the highest shoot length (3.0 ± 0.73) was obtained directly (without an intervening callus phase) within 4 weeks of culture. Further shoot elongation was achieved on medium containing 4.40 μM BA. The elongated micro shoots were rooted on MS medium fortified with 1.97 μM indole-3-butyric acid. The regenerated plantlets with roots were successfully hardened on earthen pots after proper acclimatization under greenhouse conditions. This new efficient regeneration method provides a solid foundation for genetic manipulation of maize for biotic and abiotic stresses and to enhance the nutritional values.

1. Introduction

Maize (*Zea mays* L.) is a staple cereal crop around the world, and is so because of its importance as human food, animal feed and biofuel. There is a constant increase in the demand for maize globally and more predominantly in Asia [24]. In addition to being an economically important crop, maize is also a model plant for research. Malnutrition has long been recognized as a major public health problem in developing countries, including those where maize is used as staple food. Maize as a versatile crop of importance across the world will continue to play a leading role in determining the future of

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crop improvement. Maize yield and production is frequently insufficient due to abiotic stress such as drought, aluminum toxicity or scarcity of nutrients and biotic stress such as pests, weeds and diseases [5,18]. The increasing demand for maize offers chances to develop efficient, quick and reproducible transformation protocols for the important maize local varieties addressing complex traits such as grain quality and abiotic stress tolerances [6,13]. The real challenge that we face today is to develop technologies that will boost food production under a range of stressful conditions and with limited crop land to meet human demand. However, an efficient plant tissue culture procedure with high regeneration frequency is a prerequisite for most of the approaches. The regeneration of maize plants from immature embryos was first reported in1975 [4]. Consequently, plant regeneration from calli induced from split node explants [12], anthers [23], glume calli [21], immature inflorescences [11], immature tassels [16,19], leaf segments [2,15], seedling segments [17], shoot tips [8,10,26], shoot apical meristems [25] and protoplasts [7] has also been reported successfully. Maintenance and production of immature embryos of maize are challenging and time consuming and they require a well-equipped greenhouse and laborious artificial pollination system. Furthermore, dry mature seeds are available in plenty, throughout the year and they are amenable to tissue culture [3].

Till date no reports are available describing direct shoot organogenesis in maize using split node explants. This paper for the first time reports reliable and an efficient method for *in vitro* direct shoot organogenesis and plant regeneration from split nodal ex-plants of maize derived from seedling. This new plant regeneration method will open up new avenues in plant tissue culture and genetic engineering methodologies in maize.

2. Materials and methods

2.1. Plant material and seed sterilization

Healthy and mature viable seeds (*Zea mays* L.) (HQPM-5) were collected from national seeds corporation limited, Hyderabad, India. Approximately 2 gm of mature seeds were initially surface sterilized with 70% ethanol for 2 min, followed by 0.1% mercuric chloride ($HgCl_2$) for 15 min. The sterilized seeds were rinsed six times with sterilized water to remove the surfactants and soaked in sterilized distilled water for 48 h. All the steps above were performed under the laminar flow. All the plant growth hormones used in this study were purchased from Duchefa Biochemie.

2.2. Seed germination and collection of split node explants

For seed germination, surface sterilized seeds were cultured on Murashige and Skoog medium [9] lacking plant growth regulators and incubated in the light (25 ± 2 °C with 50 μmol m^{-2} s^{-1}) at 27 °C. The nodal region of 7–10-day old seedlings, about 0.5 cm above and below the node, was excised and split longitudinally into two halves. The split pieces were positioned, wounded surface down, on regeneration medium containing different concentrations of BA (2.20, 4.40, 6.60, 8.80,11.0 and 13.2 μM)or Kn (2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM) and incubated under 16 h light (25 ± 2 °C with 50 μmol m^{-2} s^{-1}) at 25 ± 2 °C.

2.3. Plant regeneration from split node explants

The split node explants were transferred onto regeneration medium containing 30 gl^{-1} sucrose and 8 gl^{-1} agar augmented with various concentrations of BA (2.20, 4.40, 6.60, 8.80,11.0 and 13.2 μM) or Kn (2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM). The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were maintained at 25 ± 2 °C under a 16-h photoperiod. The regenerating shoots (4 week old) were transferred to MS medium supplemented with different concentrations of BA (2.2, 4.4, 6.6 and 8.8 μM) for further elongation and cultured for 2 weeks onto this medium.

2.4. Rooting, acclimatization and green house transfer

Microshoots regenerated from split node explants (2–3 cm) were excised and rooted on MS medium supplemented with different concentrations of indole-3-butryic acid (IBA) (0.98, 1.97, 2.95 and 3.93 μM). *In vitro* regenerated plantlets obtained after 10–15 days of culture on rooting medium were carefully removed from the culture bottles and washed under running tap water until agar was removed completely. These plantlets were transferred to earthen pots containing vermiculite and perlite in a 1:1 ratio followed by acclimation in the greenhouse (28 °C day, 24 °C night, and 80–90% RH).

Plant growth regulators (PGRs) used in the study were added prior to autoclaving the medium. The explants (split nodes) were inoculated in petri dishes (90 × 20 mm) containing MS medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India). All media pH were adjusted to 5.8 with 0.1 N NaOH before adding agar and sterilized at 121 °C for 15 min. All cultures were maintained at 25 ± 2 °C under white fluorescent light (50 μmol m^{-2} s^{-1}) with 16 h photoperiod. For culture establishment and multiplication from split node explants, 10 explants were used in each of two replicates for each treatment and the experiment was repeated twice. Data pertaining to number of shoots per culture, shoot regeneration percentage, and mean shoot length were recorded after 4 weeks. The data were analyzed statistically using Duncan's multiple range test (DMRT).

3. Results and discussion

3.1. Culture establishment

The study described here was under taken to examine tissue culture response of split node explants of maize for direct organogenesis with the aim to establish an efficient and reproducible regeneration protocol for further genetic transformation studies. Freshly harvested nodal explants collected off 7–10 day old seedlings were found to serve as suitable sources of explants for culture establishment (Fig. 1a). Initially collected nodal explants were split longitudinally into two halves and placed wounded surfaces on MS media with different concentrations of BA (2.20, 4.40, 6.60, 8.80, 11.0 and 13.2 μM) (Table 1) (Fig. 1b). When split nodal explants were incubated on MS media with 8.80 μM, 80% of explants responded after 10–15 days following culture. When split node explants were placed on medium with different concentrations of Kn (2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM) (Table 1), response of

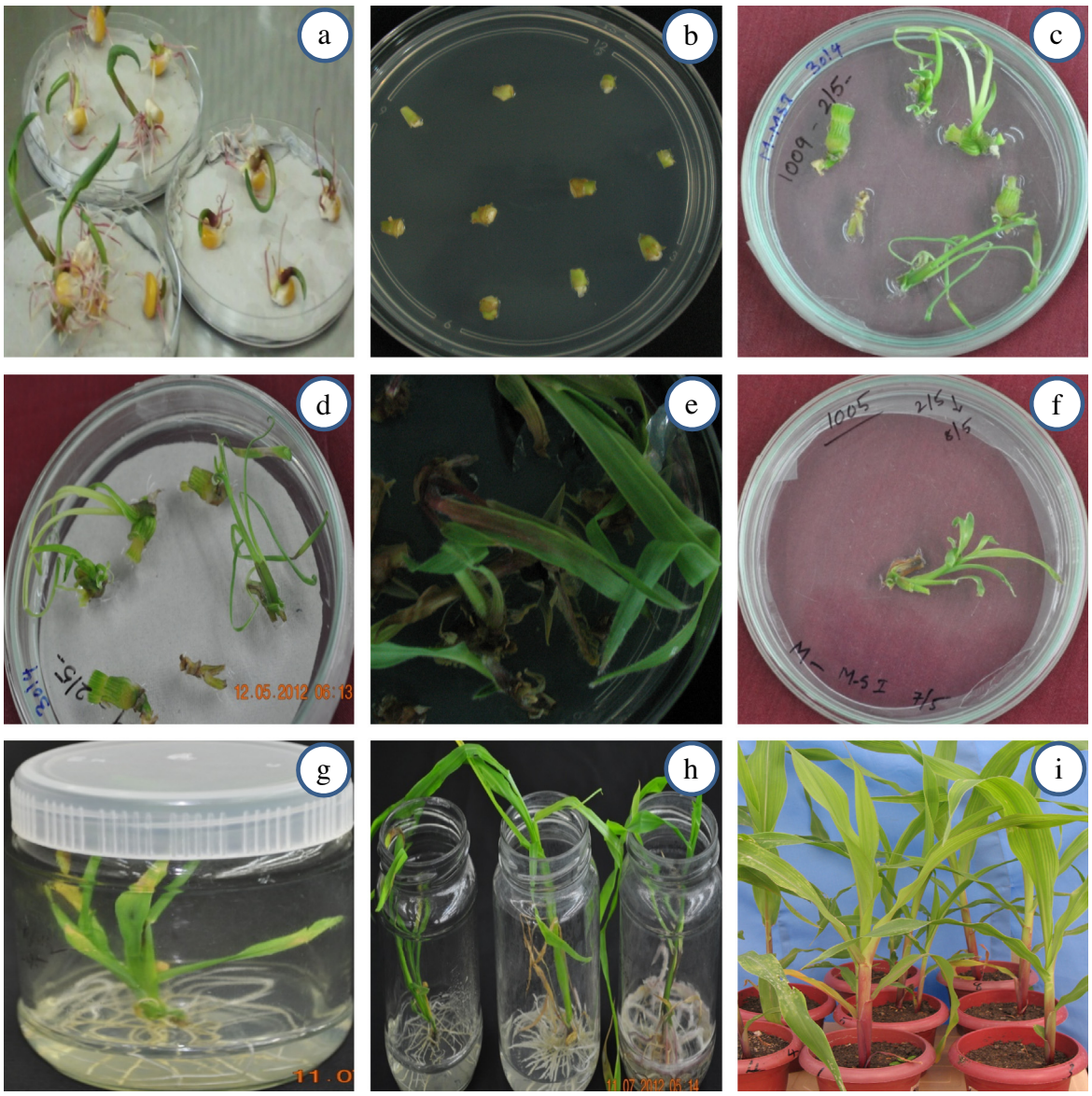

Figure 1 Plant regeneration from split node explants of maize (a) 7 day old *in vitro* grown seedlings (b) longitudinally split nodes placed on medium containing 8.80 µM BA (c) direct regeneration of multiple shoots from split node explants on MS medium containing 8.80 µM BA (d) elongation of regenerated shoots on MS medium containing 4.40 µM BA (e) shoots with fully expanded leaves (f) young and healthy regenerated plantlet (g–h) plantlets with well-established roots on MS medium containing 1.97 µM IB (i) *in vitro* raised plants under greenhouse conditions.

regeneration is very low but shoots were regenerated and mean number of shoots was lower (1–1.35) than that obtained with BA per explant (Table 1). BA in the culture medium significantly increased the regeneration response and shoot elongation.

3.2. Shoot regeneration and elongation

Recent reports described the regeneration of maize plants from calli (embryogenic and organogenic) derived from split node ex-plants of maize [12]. First time, here we report the direct regeneration of shoots from split node explants without intervening callus phase. For direct shoot regeneration from split node explants, different concentrations of BA or Kn were tested. The optimum concentration of BA; 8.80 µM produced 12.0 shoots with a greater length of 3.0 cm (Table 1) (Fig. 1c). The efficiency of direct shoot regeneration varied with PGR concentrations used in the study. Shoot initials developed from the cut ends of split node explants that further modified into shoots (Fig. 1c). This was a common morphogenic response witnessed for all regenerating cultures. BA at concentrations of 8.80 and 11.0 µM induced a significantly higher number of multiple shoots (12.0 and 9.0 shoots/explant respectively) (Table 1). However, the percent of such responding cultures was more at 8.80 µM BA (80%) as compared to 11.0 µM BA (50%). The regenerated shoots attained a mean length of 3.0 ± 0.73 and 2.8 ± 0.31 at concentrations 8.80 and 11.0 µM of BA respectively (Table 1). Kn at 6.97 and 9.29 µM induced

Table 1 Effect of BA or Kn on plant regeneration from split node ex-plants of maize.

Growth regulator (μM)		Response (%)	Morphogenic response (shoot–callus)	Shoots No./explant ± S.E.	Shoot length (cm ± S.E.)
BA	Kn				
2.20	–	20	C	–	–
4.40	–	35	S + C	6.0 ± 0.70[c]	2.2 ± 0.34[c]
6.60	–	50	S	7.0 ± 0.82[c]	2.5 ± 0.39[c]
8.80	–	80	S	12.0 ± 1.15[d]	3.0 ± 0.73[d]
11.0	–	50	S	9.0 ± 1.05[d]	2.8 ± 0.31[b]
13.2	–	35	S	6.2 ± 0.97[d]	2.2 ± 0.35[c]
	2.32	20	C	–	
	4.65	40	S + C	3.0 ± 0.35[a]	1.5 ± 0.34[c]
	6.97	40	S	4.0 ± 0.44[a]	2.6 ± 0.37[c]
	9.29	35	S	5.0 ± 0.56[b]	2.2 ± 0.54[d]
	11.6	30	S + C	3.0 ± 0.32[a]	1.5 ± 0.25[a]
	13.9	0	C	–	–

S, shoot; C, callus.
Values are mean of 40 explant ± S.E.
In each column mean followed by different superscripts in a column were significantly different ($p < 0.05$) according to DMRT.

multiple shoot induction from split node explants with 40 and 35% of culture response respectively (Table 1). At these concentrations, the mean number of shoots per explant was 4.0 ± 0.44 and 5.0 ± 0.56 and that attained an average length of 2.6 ± 0.37 and 2.2 ± 0.54 respectively (Table 1). At lower levels BA (2.20 μM) or Kn (2.32 μM) caused a decrease in percent of responding cultures (20%) (Table1). Higher level of BA (13.2 μM) also induced shoots but resulted in a decrease in percent of responding cultures (35%) but higher levels of Kn (13.9 μM) induced callusing from the bases of split node cultures (Table 1). At the concentrations of BA (4.40 μM) or Kn (4.65, 11.6 μM) resulted in callus formation accompanied with shoot formation (Table 1). Shoot multiplication and elongation was also achieved by sub culturing the shoot clumps on MS with 4.40 μM BA (Fig. 1d and e) (Table 2). It has been concerned that the regeneration potential of an explant is influenced by the type and/or concentration of cytokinins, BA or Kn in the medium, and that the number of shoots per explant could be increased by employing the balance of PGRs in the medium [22]. Thus it is established that BA (8.80 μM) was overall the most effective cytokinin in terms of direct shoot organogenesis and multiple shoot induction from split node ex-plants of maize. Shoots regenerated were healthy and strong (Fig. 1f). Delay in subculture resulted in yellowing and drying of shoots, therefore cultures have to be subcultured after a regular interval. Rate of shoot multiplication and elongation achieved in the present study is high, compared to other explants used in previous studies. The present study confirms the significance of the cytokinins (BA or Kn) for the direct shoot regeneration in maize. These findings are similar to the reports described in other plants (*Adhatoda vasica* and *Adhatoda beddomei*) using split node explants [1,14,20].

3.3. Rooting and acclimatization

Elongated shoots were transferred to root induction medium containing different concentrations of IBA (0.98, 1.97, 2.95 and 3.93 μM). Among the tested concentrations MS medium supplemented with 1.97 μM IBA induced more number of roots (15.6 ± 0.20) with highest root length (6.5 ± 0.40) (Table 3). These results are in accordance with *in vitro* rhizogenesis studies in maize [8]. All regenerated shoots showed 80% rooting efficiency on IBA medium (Fig. 1g and h) (Table 3). *In vitro* rooted plantlets were gently washed under running tap water to remove adhering medium and transferred to plastic pots containing a mixture of vermiculite and perlite (1:1), followed by transfer to greenhouse for further growth. New leaves emerged in a week and healthy plants were obtained

Table 2 Effect of BA on elongation of *in vitro* regenerated shoots of maize.

Growth regulator BA (μM)	Response (%)	Shoot length (cm ± S.E.)
2.2	55	2.2 ± 0.31[a]
4.4	75	4.5 ± 0.37[b]
6.6	65	3.0 ± 0.63[c]
8.8	60	2.8 ± 0.31[a]

Values are mean of 40 explant ± S.E.
In each column mean followed by different superscripts in a column were significantly different ($p < 0.05$) according to DMRT.

Table 3 Influence of different IBA on rooting performance of *in vitro* grown shoots of maize.

Growth regulator IBA (μM)	Response (%)	Average no. of roots	Average root length (cm ± S.E.)
0.98	40	2.0 ± 0.21[a]	2.6 ± 0.20[a]
1.97	80	15.6 ± 0.20[c]	6.5 ± 0.40[c]
2.95	50	6.6 ± 0.13[b]	4.2 ± 0.26[b]
3.93	40	4.0 ± 0.07[a]	2.8 ± 0.11[a]

Values are mean of 40 explant ± S.E.
In each column mean followed by different superscripts in a column were significantly different ($p < 0.05$) according to DMRT.

within one month from the transfer to the greenhouse (Fig. 1i). The survival rate under greenhouse condition was 70%.

4. Conclusion

To our knowledge, this is the first report in maize with a protocol for direct organogenesis from split node explants. In this investigation, we have used split node explant derived from 7 to 10 day old grown seedlings and achieved reliable and efficient direct regeneration without the intervening phase of callus. Direct shoot organogenesis achieved from split node explants could be useful for establishment, rapid propagation and genetic manipulation studies of maize plants.

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