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Increased morphogenetic competence in *Cuminum cyminum* L. mediated through micronutrient manipulation

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Abstract
Cumin is an important medicinal, aromatic and a spice plant. It is one of the earliest known minor spices used by mankind. It is an annual herb belonging to family Apiaceae. India is the largest producer, consumer and exporter of cumin where it is extensively cultivated in Rajasthan and Gujarat as a rabi crop. It is highly susceptible to *Fusarium* wilt, which causes considerable damage to the crop. Disease resistant cumin varieties could be developed using genetic transformation techniques, for which an efficient plant regeneration protocol is a pre- requisite. To develop an efficient and improved protocol for regeneration of cumin through micropropagation the effect of sodium molybdate (Na_2MoO_4) on shoot morphohgenesis from cotyledonary node explants was investigated. The explants were inoculated on MS medium supplemented with Kn (0.5mg/l), this medium was termed as 'Induction Medium' as supported the induction of shoot buds from cotyledonary node explants. These shoot buds were sub cultured on MS medium with same level of kinetin (0.5 mg/l) for their further multiplication, hence this medium was termed as 'Proliferation Medium'. The Induction and Proliferation Medium were supplemented with different levels of Na_2MoO_4 (0, 1.03*, 5.15, 10.3, 20.6, 51.5 µM). Sodium molybdate improved shoot morphogenesis in *Cuminum cyminum*. Maximum differentiation of shoot buds was observed when Na_2MoO_4 level was raised to 20.6 µM in the induction medium and reduced to 1.03 µM in the proliferation medium.

Keywords: cumin, induction, sodium molybdate, cotyledonary node, seed spice

Introduction
India is blessed with a rich heritage of spice crops spread across different agro-ecological regions. Out of 109 spices listed by ISO, India produces as many as 75. Over the years the market for Indian spices has increased and India dominates the world trade by contributing almost 45% of the global spice export. *Cuminum cyminum* L. is an important seed spice belonging to family Apiaceae. It is cultivated extensively in Rajasthan and Gujarat where it is grown as a rabi crop and is a major cash crop of India. Conventional breeding techniques have been of immense value for cumin genetic improvement but utilize huge amounts of seeds, which results in the wastage of the crop significantly. Moreover, natural cross-pollination results in a high level of heterogeneity in seed population. Disease and pests also limit their productive area, yield and poses a great threat to their cultivation. Slow initial growth of the crops face severe competition from weeds. Zeeri (*Plantago pumilla*), a serious weed of cumin, cannot be distinguished from the cumin plant upto the stage of flowering and results in reduction of volatile oil content and test weight of cumin. Cumin is highly susceptible to *Fusarium* wilt, which causes considerable damage to the crop. Disease resistant cumin varieties could be developed using genetic transformation techniques, for which an efficient plant regeneration protocol is a pre- requisite. Previous reports on indirect regeneration through somatic embryogenesis or organogenesis (Tawfik and Noga, 2001, 2002) [13, 14]. Callus mediated regeneration has limited use in transgenic plant development as there are problems in transgene inheritance and stability of expression in transgenic plants regenerated through long term callus phase (Choi *et al.*, 2000) [2]. The selection of proper media formulation plays a crucial role in establishing an efficient tissue culture system for plant regeneration. The nutrient levels in the medium have profound effects on embryogenic callus induction and plant regeneration (Dahleen, 1995; Ramage and Williams, 2002) [3, 10]. However, since plants differ in their response to nutrients when grown *in vitro*, there is clearly a need to determine the

effects of essential plant nutrients on in vitro plant growth. At a practical level, an understanding of optimal nutrient concentration may lead to increased growth rates and promote plant morphogenesis *in vitro*.

In the present study, we report an efficient direct shoot regeneration protocol for cumin which could be used for development of transgenic plants. Also, we optimized micronutrient molybdenum requirements for shoot regeneration in cumin.

Materials and Methods

A. Establishment of aseptic seedlings and explant preparation

Seeds of *Cuminum cyminum* L. var. RZ 19 were procured from NRCSS, Ajmer. The seeds were rinsed with 20% (v/v) Extran (Merck, India) followed by 3-4 washings with sterile distilled water. These were then surface-sterilized with 0.1% $HgCl_2$ aqueous solution for 3 min and rinsed with three changes of sterile distilled water. MS medium with 3% (w/v) sucrose and solidified with 0.8 % (w/v) agar (Qualigens, bacteriological grade), pH adjusted to 5.8 before autoclaving at 121^0C and 1.2–1.3 kg/cm^2 pressure for 20 min was prepared for germination of seedlings. Five seeds were kept in a single flask (100 ml 'Erlenmeyer' with 40 ml medium in each). All the cultures were incubated in growth chamber at a temperature of 26^0C ± 1^0C and 16 h photoperiod and light intensity of 25 µmol/m^2/s provided by white fluorescent tubes. Cotyledons were excised aseptically from 8-10 day old aseptically grown seedlings and the remaining cotyledonary node was used as an explant.

B. Culture media

The cotyledonary node explants were inoculated on the MS medium (Murashige and Skoog, 1962) supplemented with 0.5mg/l Kn and sucrose 3 %(w/v). This was considered to be control induction medium having 1.03 µM of Na_2MoO_4 in MS basal medium. The levels of Na_2MoO_4 (0, 1.03*, 5.15, 10.3, 20.6, 51.5 µM) (Table 2; Fig. 1a-d). Five flasks (100 ml 'Erlenmeyer' with 40 ml medium in each) were prepared for each treatment having three explants per flask. Visual observations were made for 4 weeks. Percentage response was calculated by dividing the total number of responding explants by total number of explants inoculated. Shoots buds induced from the explant were excised after 4 weeks and sub-cultured on normal proliferation medium NPM (MS medium supplemented with 0.5mg/l Kn and 1.03 µM of Na_2MoO_4 as present in normal MS medium) and on modified proliferation medium MPM (MS medium supplemented with 0.5mg/l Kn and varied levels of Na_2MoO_4 (0, 1.03*, 5.15, 10.3, 20.6, 51.5 µM). Weekly observations were recorded for 4 weeks. Morphogenic competence was observed taking into account the quality and number of shoots per explant.

C. Rooting and Acclimatization

Shoots of appropriate length were excised and transferred on to rooting medium consisting of full strength MS medium supplemented with 0.5mg/l IBA. Plantlets with well developed shoot and root systems were carefully taken out and washed with tap water to remove agar. These plantlets were then transferred to earthen pots containing garden soil and organic manure (1:1). Humidity was maintained initially by covering the pots with polythene bags.

Results & Discussion

First, a direct regeneration protocol was optimized from the cotyledonary node explants of cumin and then the effect of sodium molybdate was studied on regeneration. The explants (cotyledonary node) were excised from 8-10 day old aseptically grown seedlings and cultured on kinetin/ BAP (0.3- 5 mg/l) supplemented medium. Frequency (%) of explants showing shoot regeneration and the rate of multiplication depended on the type and concentration of growth regulators. When treated with BAP, 60-75% explants showed regeneration response which was higher (upto 95%) in case of kinetin supplemented medium. Formation of shoots from the explant occurred on all the hormonal concenterations tried, but medium supplemented with Kn (0.5 mg/l) promoted maximum differentiation of shoots (Table 1). Higher levels of kinetin (1-5 mg/l) resulted in formation of lesser number of shoots from the explant. Thus, MS medium supplemented with Kn (0.5 mg/l) was optimized for the induction of shoot buds and was referred to as Induction Medium. The shoot buds induced on this medium when sub-cultured on the same hormonal combination proliferated to give an average of 14 shoots and this was termed as Proliferation Medium. Medium supplemented with BAP was not found to promote shoot regeneration. Thus, kinetin proved to be superior than BAP for regeneration in cumin. Although BAP is related to Kn in structure and is regarded to be much more effective and stable than Kn. In our study, Kn scores over BAP in caulogenic response elicitation. Ebrahimie *et al.* (2006) [6] also reported that addition of BAP to the regeneration medium suppressed direct regeneration pathway and induced callus formation in cumin. The superiority of Kn over BAP in elicitation of *in vitro* caulogenic response has also been observed in *Phyllanthus amarus* (Bhattacharyya and Bhattacharya, 2001) [1].

The development of shoots from cotyledonary node explants (having axillary buds) is based on the principle of removing apical dominance leading to axillary buds to proliferate (Dunston and Thorpe, 1986) [5]. Such a phenomenon is commonly observed in plants *in vivo* or cultured under *in vitro* conditions. This is largely due to main growing shoot exerting a control over plant developmental processes such as axillary bud growth, orientation of laterals etc. The response, besides nutrition and other factors, is governed pre-eminently by phytohormones (Tamas, 1995) [12]. Theoretically, the advantage of direct shoot regeneration from meristematic explants over other regeneration systems is that plants may be obtained from any genotype rather than from only those that regenerate from callus culture (Zapata *et al.*, 1999; Molinier *et al.*, 2002) [15, 8]. Meristems are highly reactive cells and plants regenerated from them generally show no variation in genotypic and phenotypic characters (Saeed *et al.*, 1997) [1]. In relation to callus culture, shoots multiplied by apices or axillary buds have genetic stability and show little new variability during selection (Hu and Wang, 1983) [7].

To study the effect of sodium molybdate on regeneration response from the cotyledonary node, the explants were cultured on shoot bud induction medium. Initially, the explants became swollen and multiple shoot primordia emerged from them after 2 weeks of culture. Shoots buds formed were normal and healthy. Varying the level of sodium molybdate in the basal medium highly influenced the number of shoot buds and the percent of responding explants (Table 2). Induction of primary shoots was seen on the medium devoid of Na_2MoO_4. The shoots were vitrified, green and watery and failed to proliferate when sub-cultured on the same medium. On control, (basal medium with normal level of molybdate) an average of 4-5 normal shoot buds were

induced from each explant. There was a concommitant increase in number of shoot buds with increasing concentration of molybdenum till the level for mineral was optimized at 20.6 µM (which is 4 times higher than the MS level of Na_2MoO_4). Shoot buds formed on molybdenum supplemented medium were sub-cultured on proliferation medium (NPM and MPM) (Table 2). Maximum differentiation of shoot buds was observed when Na_2MoO_4 level was raised to 20.6 µM in the induction medium and reduced to 1.03 µM in the proliferation medium. (Fig.1 a-e). The number of shoots was double than the control cultures. Shoot buds induced on 20.6 µM when sub-cultured on the same medium i.e., MPM, proliferated to form an average of 10 shoots, which was comparable to control. Higher levels of sodium molybdate did not support shoot morphogenesis. Healthy shoots were rooted successfully on MS medium supplemented with 0.5 mg/l IBA and plantlets with well developed root and shoot systems were transferred to field. (Fig. 2 f-g).

Na_2MoO_4 is the source of Molybdenum in the medium. Molybdenum is primarily related to nitrogen metabolism and purine breakdown as a part of enzyme dinitrogenase, nitrate reductase and xanthine dehydrogenase. It is an essential part of oxidase that converts abscisic acid aldehyde to abscisic acid. Dahleen and Bregitzer (2002) [4] found no significant difference in regeneration response of barley explant to Na_2MoO_4 concentration except at very high and low level. On the other hand we found that increasing the molybdenum level to 4 times of basal MS improved the regeneration response in cumin. Although higher levels upto 5.15 µM was not supportive for regeneration in our study as well. Legumes need more molybdenum than other crops, such as grass or corn, because the symbiotic bacteria living in the root nodules of legumes require molybdenum for the fixation of atmospheric nitrogen. If insufficient molybdenum is available nodulation will be retarded and the amount of nitrogen fixed by the plant will be limited. If other factors are not limiting the amount of molybdenum will determine the amount of nitrogen fixed by the plant. Increasingly vigorous plant growth, higher protein contents and greater buildup of nitrogen in the plant and soil accompany nodulation and symbiotic microbial activity.

Table 1: Shoot bud formation from cotyledonary node explants of *C. cyminum* cultured on MS medium supplemented with Kn/BAP. (Number of explants = 50)

Kn/BAP in primary culture (mg/l)	Mean number of shoot buds / explant ± S.D.	Kn/BAP in second stage subculture (mg/l)	Mean number of shoot buds / explant ± S.D.
Kinetin			
0.3	4.3 ± 0.8(80)	0.3	9.1 ± 0.6
		0.5	7.8 ± 1.4
0.5	6.1 ± 1.2(100)	0.3	10.1 ± 1
		0.5	14.1 ± 0.9
1	4.1 ± 1.1(90)	0.3	6.6 ± 0.4
		0.5	6.9 ± 1.1
3	3.7 ± 0.9(80)	0.3	4.9 ± 1.2
		0.5	4.2 ± 1
5	2.4 ± 0.4(80)	0.3	3.2 ± 1.1
		0.5	2 ± 0.8
BAP			
0.3	2.1 ± 0.6(70)	0.3	2.1 ± 0.8
		0.5	2 ± 0.4
0.5	2.9 ± 1.1(70)	0.3	3.1 ± 0.6
		0.5	3 ± 1.2
1	3.1 ± 1(75)	0.3	3.7 ± 0.8
		0.5	3.2 ± 0.7
3	2.7 ± 0.5(70)	0.3	4.8 ± 0.9
		0.5	4 ± 0.6
5	1 ± 0.8(75)	0.3	2 ± 0.4
		0.5	1.6 ± 0.8

Table 2: Effects of Na_2MoO_4 on shoot bud formation from cotyledonary nodes of *C. cyminum* cultured on MS medium supplemented with Kn (0.5 mg/l) (Number of explants = 50)

Na_2MoO_4 (µM) in primary culture	No. of shoot buds/explant Mean ± S.D.	Na_2MoO_4 (µM) in second stage subculture	No. of shoot buds/explant Mean ±S.D.
0	1.2 ± 0.4	0	0
		1.03[a]	3.2 ± 1.1
1.03[a]	3.3 ± 0.5	1.03[a]	14.4 ± 1.7
5.15	4.3 ± 0.8	1.03[a]	7.6 ± 1.1
		5.15	3.4 ± 1.1
10.3	5.4 ± 1.1	1.03[a]	9.8 ± 1.1
		10.3	6.7 ± 1
20.6	6.1 ± 1.2	1.03[a]	21.6 ± 1.5
		20.6	9.7 ± 0.9
51.5	3.4 ± 1.1	1.03[a]	4.1 ± 0.5
		51.5	2.6 ± 2

[a] Level of Na_2MoO_4 in MS medium

Fig 1: Shoot bud induction and proliferation from cotyledonary nodes of *Cuminum cyminum* L. cultured on MS medium supplemented with Kn (0.5 mg/l) and various levels of Na_2MoO_4
(a). Without Na_2MoO_4
(b). 1.03 µM Na_2MoO_4
(c). 20.6 µM Na_2MoO_4
(d). 1.03 (induced on similar medium)
(e). 1.03 (induced on 20.6 µM Na_2MoO_4)
(f). Rooting of regenerated shoots on MS + IBA (0.5 mg/l)
(g). Field transferred plant

Conclusion

In this study, a simple, reproducible and genotype-independent protocol for direct regeneration of shoots from cotyledonary nodes of cumin has been presented. The results obtained in the present study could be of enormous significance that can be coupled with an efficient gene transfer method. The present study also reports optimization of levels of inorganic micronutrients so as to increase the regeneration potential of cumin. The modifications of nutrients have been studied both at induction and proliferation stages and highly improved regeneration was recorded at optimized levels.

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