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HIGH BORON LEVEL IMPROVES CALLUS INDUCTION AND PLANT REGENERATION IN *HORDEUM VULGARE* (L.)

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The present study reports that the increased level of boric acid in the induction medium enhanced shoot bud induction and plant regeneration in Indian Barley cultivars. Primary cultures were raised on MSB_5 medium supplemented with 20.7 µM picloram and subsequently subcultured and regenerated on medium with 12.47 µM & 0.2 µM picloram respectively. Concentration of boric acid was modified at different stages of culture. Higher level of boric acid in the induction medium not only improved shoot but induction but also plant regeneration in later stages of cultures. Regeneration frequency of 100% with 3.4 folds more number of plantlets was achieved when H_3BO_3 concentration in the induction was raised to 1.0 mM (10 times than in MS) and reduced to 0.1 mM (Normal MS level) in the subculture and regeneration medium. The increased level of H_3BO_3 in the induction medium also proved better for other recalcitrant Indian varieties.

Key-words: Barley,Boric acid, Callus induction, Picloram, Plant regeneration.

Introduction

The importance of Barley for malting and brewing makes it a prime target for tissue culture and genetic transformation studies. Barley tissue culture dates back to 1970 when Norstog[1] for the first time cultured immature embryos. Since then regeneration has been obtained from various explants including immature and mature embryos, seeds, immature inflorescences, leaves, coleoptile, nodes and apical meristem[2-11]. Despite the various reports on *in vitro* culture of barley, the difficulties encountered in obtaining the desired response are numerous and the plant has been considered recalcitrant. A nagging problem is that the conditions suitable for plant regeneration in one cultivar fail to produce plants in another cultivar of the same species[12-13]. Most of the studies are confined to the responsive spring type genotypes and little work has been done on non-responding winter varieties. A survey of published literature shows that MS or B_5 medium[14-15] has been used most frequently for cereal tissue culture. It has been reported that a particular medium composition may not be optimal for the culture of various cultivars as there occurs interaction between mineral nutrients and other factors including the genotype and the stage of culture[2, 16-25].Various modifications of the basal medium have been proposed for callus induction and plant regeneration in wheat[26-29], rice[30-36], sorghum[37-38], finger millet[39] and rye[40]. Bregitzer *et al.*[2,41], Dahleen and Bregitzer[42], Chauhan and Kothari[43], Yadav *et al.*[44], Haque[45], Chauhan[46] investigated the effect of different nutrients on barley culture. In studies made by Dahleen[47], Bregitzer *et al.*[41], Castillo *et al.*[48], Dahleen and Bregitzer[42] optimized concentration of micro- nutrients like copper, iron and cobalt in the basal medium resulted in greater efficiency of barley cultures to regenerate green plants. The present investigation was carried out to optimize the level of boric acid

in the basal medium for efficient shoot-bud formation and high plant regeneration in Indian winter barley.

Material and Methods

Immature caryopses of barley (*Hordeum vulgare* L. BL-2) were procured from Agriculture Research Station, Durgapura, Jaipur, Rajasthan. These caryopsis were surface sterilized by soaking for 30 seconds in 70% (v/v) ethanol and then for three minutes in 0.1% aqueous solution of mercuric chloride followed by three rinses in sterile distilled water. Immature embryos were removed from these surface sterilized caryopses and cultured with their embryonal axis in contact with the medium. MSB_5 medium (MS medium[14] with B_5 vitamins[15]) supplemented with 20.7 µM picloram was used as basal induction medium for culture. The pH of medium was adjusted to 5.8 and 0.8% bacteriological grade agar (Qualigens, India) was used as gelling agent. The medium was autoclaved at 121°C and 1.06 kg/cm^2 pressure for 15 min. All the cultures were maintained at 26±1°C under a 16 h light and 8 h dark cycle with a light intensity of 24 µ mol min^{-2} s^{-1}. The concentration of H_3BO_3 was modified in the induction medium to study its effect on callus induction. After 6 weeks the leaves and roots of germinating embryos were removed and the callus was subcultured on MSB_5 medium supplemented with 12.47 µM picloram. From each of the treatment half of the cultures were subcultured on medium with normal concentration of H_3BO_3 and the other half on medium with modified concentration of H_3BO_3 as was in the induction medium. After 4 weeks of subculture, the cultures were regenerated on MSB_5 medium supplemented with 0.2 µM picloram. Cultures that were subcultured on medium with normal concentration of H_3BO_3 were regenerated on regeneration medium with normal concentration of H_3BO_3. On the other hand, cultures that were subcultured on modified concentration of H_3BO_3 were regenerated on (a) regeneration medium with normal concentration of H_3BO_3 and on (b) regeneration medium with modified concentration of H_3BO_3 as was in the subculture medium. Thus the effect of boric acid was studied at induction, subculture and regeneration level.

Results and Discussion

After 6 weeks of culture on induction medium, Barley immature embryos produced watery and translucent callus. Hard compact morphogenic region was enclosed in it. This region produced visible shoot buds after first subculture that formed shoots on regeneration medium. Effect of modified concentration of H_3BO_3 was not apparent at induction level, however, it affected the development in later stages.

Elevated concentrations of H_3BO_3 increased the shoot-bud forming capacity and plant regeneration efficiency of the cultures (Table 1). Higher percentage response along with more number of shoot buds was observed when the induction medium was modified for H_3BO_3 concentration and subculture medium remained normal. Comparatively poor response was observed when both the induction and subculture medium had higher concentration of H_3BO_3 yet all the treatments with higher H_3BO_3 were better responding than the control. With 0.1 mM H_3BO_3 in the subculture medium, the percentage of the responding cultures as well as the number of shoot buds formed per explant increased with an increase in H_3BO_3 concentration in the induction medium [Fig 1. A(i-iii)]. The percentage of shoot bud forming cultures was raised to 100% when the induction medium had 0.5 mM, 0.7 mM or 1 mM H_3BO_3 and the subculture medium had 0.1 mM H_3BO_3. These combinations also showed 100% regeneration frequency on 0.1 mM H_3BO_3. Among the various concentrations examined induction on 0.7 mM or 1.0 mM was considered better as the maximum number of shoot buds was formed on these concentrations.

The regeneration response was also influenced by the H_3BO_3 concentration in the induction medium. The response became better as the concentration of H_3BO_3 in the

induction medium was increased upto 1 mM, further increase in the concentration led to a decline in the regeneration (Table 2). For the number of green plants regenerated, the best treatment combination was 1 mM H_3BO_3 in the induction and 0.1 mM in both the subculture and regeneration medium [Fig 1. B-E]. This treatment combination regenerated 3.4 folds higher number of plantlet per explant with 100% of regeneration frequency. The increased level of H_3BO_3 in the induction medium not only proved better for variety BL-2 but also for other recalcitrant Indian varieties RD-2660, RD-2668, RD-2503, RD-2628, RD-2035, RD-2552, RD-2052, RD-2592, RD-2715, RD-2508 (Table 3). Thus the level of H_3BO_3 in the MS medium proves to be sub-optimal for barley culture, its higher level is required in the induction medium not only to improve shoot bud formation but also for higher plant regeneration.

Table 1: Callus induction and shoot bud differentiation from immature embryos of barley var. BL-2 in response to MSB_5 medium supplemented with modified concentrations of H_3BO_3

Concentration of H_3BO_3 in callus induction medium	Concentration of H_3BO_3 in callus subculture medium	Shoot buds formed per explants after 10 weeks Average ±S.D. (% Response)
0.1 mM#	0.1 mM#	2.21±0.78 (60)
0.2 mM	0.1 mM#	4.6±0.54 (83)
	0.2 mM	3.33±0.7 (90)
0.5 mM	0.1 mM#	5.16±0.75 (100)
	0.5 mM	3.4±0.69 (83)
0.7 mM	0.1 mM#	**6.33±0.51 (100)**
	0.7 mM	3.77±0.83 (75)
1 mM	0.1 mM#	**6.33±0.81 (100)**
	1 mM	3.2±0.63 (83)
1.5 mM	0.1 mM#	5.33±0.57 (75)
	1.5 mM	2.66±0.7 (90)

Picloram concentration was 20.7 µM in callus induction and 12.47 µM in callus subculture medium
Concentrations in normal MSB_5 medium

Similar results were obtained by Sahasrabudhe *et al*.[49] for rice, Dahleen and Bregitzer[42] for barley, Huang and Li[50] for pine and Zoglauer *et al*.[51] for tea, peanut and potato where concentration of boric acid in the basal medium proved to be sub-optimal. Sahasrabudhe *et al*.[49] obtained higher rates of regeneration through increase in boric acid concentration in the basal medium though the requirement was found to be specific for embryogenesis in different explants. High boron concentration significantly increased

Table 2: Regeneration response of barley var. BL-2 cultures initiated, subcultured and regenerated on MSB_5 medium with modified concentrations of H_3BO_3

Conc. of H_3BO_3 in callus induction medium	Shoots regenerated per explant Average ±S.D. (Percentage regeneration)		
	S_NR_N	S_MR_N	S_MR_M
0.1 mM#	1.75±0.95 (31)	1.75±0.95 (31)	1.75±0.95 (31)
0.2 mM	2.33±0.57 (75)	3±0.81 (80)	3.5±1 (80)
0.5 mM	3.33±1.03 (100)	3.25±0.5 (80)	3.4±0.54 (83)
0.7 mM	4.2±0.83 (100)	3.33±0.57 (75)	3.5±0.57 (80)
1 mM	**6±0.81 (100)**	4.33± 0.57(60)	4.33±0.57 (60)
1.5 mM	6±1 (75)	4±0 (50)	5±0 (33)

H_3BO_3
Picloram concentration was 20.7 µM in the induction, 12.47 µM in the subculture and 0.2µM in the regeneration medium.
Concentrations in normal MSB_5 medium
S_N,R_N : Subculture and regeneration medium respectively with normal (N) conc. of H_3BO_3.
S_M,R_N : Subculture medium with modified (M) and regeneration medium with normal (N) conc. of H_3BO_3.
S_M,R_M : Subculture and regeneration medium respectively with modified (M) conc. of H_3BO_3

Table 3: Regeneration response of barley varieties on medium with improved levels of H_3BO_3 as compared to the basal MSB_5 medium

Variety	Average number of plants regenerated per explants (Percentage regeneration)	
	Basal MSB_5 medium	MSB_5 medium with improved level of H_3BO_3
RD-2660	2.0 (35)	5.0(60)
RD-2668	1.0 (20)	6.5(65)
RD-2503	0	4.0(35)
RD-2628	0	3.0(40)
RD-2035	0	3.5(45)
RD-2552	0	3.0(50)
RD-2052	0	2.5(35)
RD-2592	0	7.0(65)
RD-2715	0	1.5(50)
RD-2508	0	3.5(40)

shoot proliferation in kiwifruit[52]. Dahleen and Bregitzer[42] found 0.75 mM H_3BO_3 more appropriate for barley green plant regeneration than MS concentration. According to Brdar-Jokanovi´c[53] dramatic difference in terms of boron requirements

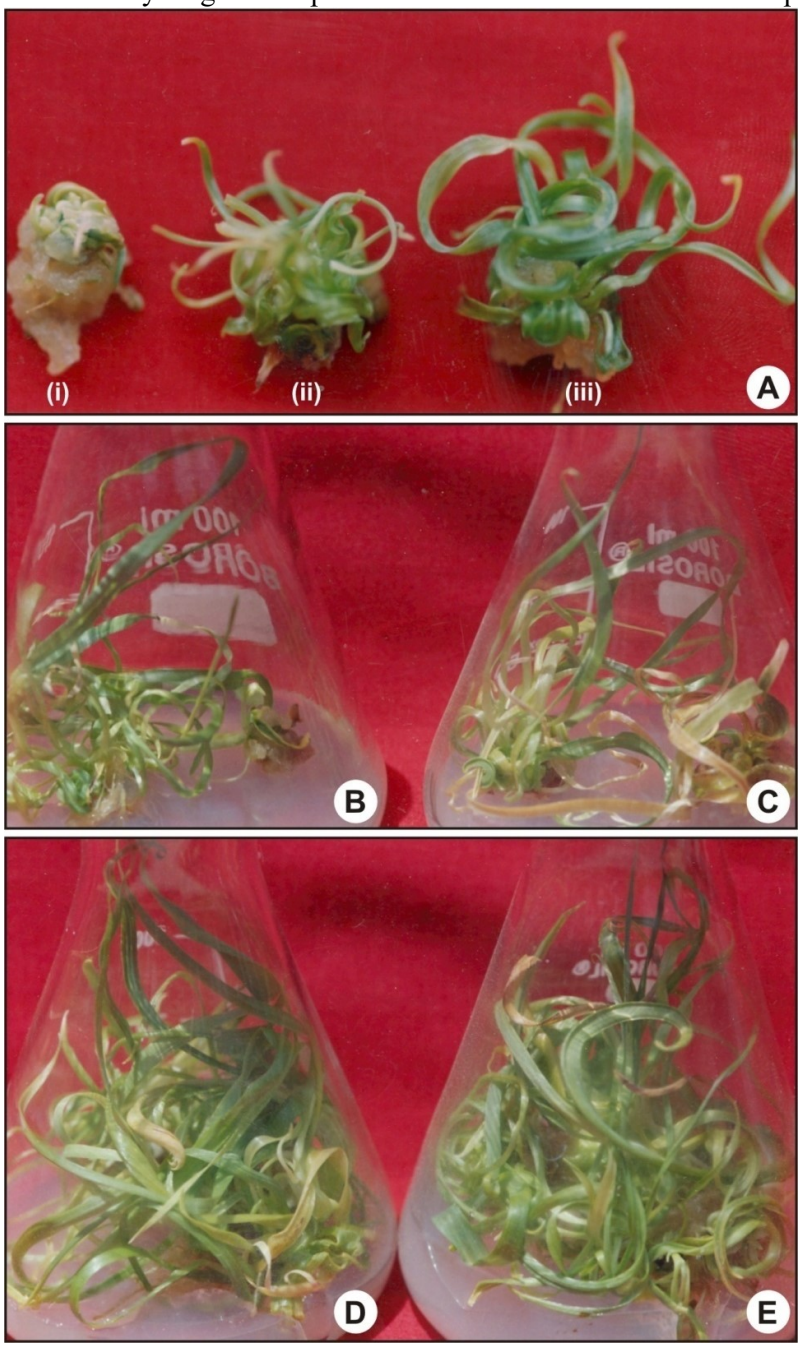

Fig 1: (A) Shoot buds formed on normal subculture medium [MSB_5 + picloram (12.47 µM)] from cultures induced on MSB_5 medium with picloram (20.7 µM) and H_3BO_3
(i) 0.1 mM (ii) 0.2 mM (iii) 1 mM
(B-E) Plant regeneration from cultures induced on MSB_5 medium with picloram (20.7 µM) and various concentrations of H_3BO_3 but subcultured and regenerated on normal medium [MSB_5 + picloram (12.47 µM & 0.2 µM respectively)]
(B) 0.1 mM H_3BO_3 i.e., Control (C) 0.2 mM H_3BO_3
(D) 0.5 mM H_3BO_3 (E) 1mM H_3BO_3

occurs between plant species, as well as the genotypes within the species.

The exact mechanism for this enhancement is not known though Boron has been reported to have significant effect on Ca metabolism [52,54]. It is involved in the structure and functioning of cell wall and membrane and thus participates in numerous ion, metabolite, and hormone transport reactions[53]. Boron plays role in plants by regulating physiological processes, improving the structural integrity of cytosolic organs, and enhancing antioxidant defense systems and thus provides stress tolerance[55]. Loomis and Durst[56] postulated various roles of boron.

Thus the present study concludes that 1 mM H_3BO_3 in the induction and 0.1 mM in both the subculture and regeneration medium is optimum for tissue culture of Indian Barley varieties.

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