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ENHANCEMENT OF PLANT REGENERATION IN RECALCITRANT INDIAN BARLEY THROUGH OPTIMIZATION OF COPPER SULPHATE IN THE MEDIUM

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Effect of copper sulphate on the morphogenetic potential of barley callus induced from immature embryos and its subsequent plant regeneration was investigated in the present study. MSB_5 medium with picloram served as the basal medium. Concentration of $CuSO_4$ was modified in the induction, subculture and regeneration medium to examine its effect on different stages of culture. Higher levels of $CuSO_4$ were found to be beneficial. Callus induction, its morphogenic potential, regeneration frequency and average number of regenerated plantlets was influenced by the level of $CuSO_4$ in the medium. Presence of $CuSO_4$ during all the stages of culture was essential for plant regeneration. Plantlets could not be regenerated from callus induced on medium devoid of $CuSO_4$.There was differential requirement of $CuSO_4$ at induction, subculture and regeneration. Almost 3.5 folds increase in the number of regenerants per explant with 100% regeneration frequency was achieved when $CuSO_4$ concentration in both the induction and subculture medium was 1 µM and 0.1 µM in the regeneration medium.

Keywords: Copper sulphate, *Hordeum vulgare*, Indian barley, Plant regeneration, Shoot bud induction.

Introduction

Barley is an important cereal extensively used as food and cattle feed and in malting, brewing and pearling. In India, it is the second most important crop of the rabi season. Genetic manipulation techniques require a highly efficient and reproducible tissue culture system for improvement of barley. Since the first report of barley culture[1],tissue culture has been reported from several explants including mature[2] and immature embryos[3], seeds[4], coleoptile[5], inflorescence[6], leaves[7], nodes[8], seedling explants[9] and apical meristem[10]. Efficient plant regeneration in barley is mostly limited to model cultivars such as Golden promise, Igri or for cultivars grown in Canada, US and Spain[11,12] while Indian cultivars lag far behind. Indian barley is considered highly recalcitrant as it is difficult to regenerate and regeneration efficiency is low making it challenging to produce healthy plants from tissue culture. Significant differences in the efficiency of callus induction, somatic embryogenesis, organogenesis and plant regeneration have been attributed to genotype variability and specific requirement of media composition and plant growth regulators[13-16]. The objective of this research was to evaluate the optimum levels of copper sulphate in the basal medium for different stages of *in vitro* culture of recalcitrant Indian barley varieties.

Material and Methods

Immature caryopses of barley (*Hordeum vulgare* L. cultivar BL-2) were procured from Agriculture Research Station, Durgapura, Jaipur, Rajasthan and surface sterilized by soaking for 30 seconds in 70% (v/v) ethanol, three minutes in 0.1% aqueous solution of $HgCl_2$ followed by three rinses in sterile distilled water. Immature embryos were removed from these caryopses and cultured with their embryonal axis in contact with the medium. MSB_5 medium based on basal

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MS medium[17] with B_5 vitamins[18] and 3% (w/v) sucrose served as the basal medium.

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 121ºC and 1.06 kg/cm^2 pressure for 15 min. All the cultures were maintained at 26±1ºC under a 16 h light and 8 h dark cycle with a light intensity of 24 µ mol min^{-2} s^{-1}. The basal medium was supplemented with 20.7 µM Picloram for callus induction. The leaves and roots of the germinating embryos were removed after 6 weeks and primary callus was subcultured on MSB_5 medium supplemented with 12.47 µM picloram. For plant regeneration, cultures were further transferred to medium with a lower concentration of picloram (0.20 µM). They were kept for 4 weeks on subculture medium and for another 4 weeks on the regeneration medium. The regenerated shoots were rooted on MSB_5 medium supplemented with 2.85 µM IAA and solidified with 0.2% (w/v) phytagel.

Specific modifications were made in the level of $CuSO_4$ in the induction medium to study the effect of copper sulphate on callus induction. Primary cultures were subcultured on medium with modified concentration of $CuSO_4$ (as per the callus induction medium) as well as on medium with the normal concentration of $CuSO_4$ (as in the basal MSB_5 medium). Both the media were supplemented with 12.47 µM picloram.

After 4 weeks, cultures subcultured on the normal concentration of $CuSO_4$ were regenerated on normal MSB_5 medium while those subcultured on medium with modified concentration of $CuSO_4$ were regenerated on medium with modified concentration of $CuSO_4$ (as per the callus induction and subculture medium) as well as on medium with the normal concentration of $CuSO_4$ (as in the basal MSB_5 medium). Thus, the effect of copper sulphate was studied at induction, subculture and regeneration level.

Table 1.: Callus induction and shoot bud differentiation from immature embryos of barley var.BL-2 on MSB_5 medium supplemented with different concentrations of $CuSO_4$

Conc. of $CusO_4$ in callus induction medium (µM)	**Shoot buds formed/explant Average ± S.D. (%Response)**	
	Subculture medium with 0.1 (µM)[#] $CusO_4$	Subculture medium with $CusO_4$ conc. as in callus induction medium
0	1.75±0.5 (56%)	1.37±0.51 (56%)
0.1[#]	2.14±0.69 (58%)	2.14±0.69 (58%)
0.2	2.6±0.54 (83%)	2.45±0.52 (91%)
0.3	2.8±0.44 (83%)	2.81±0.87 (91%)
0.4	3.2±0.83 (83%)	3.45±0.52 (91%)
0.5	3.4±0.54 (100%)	4.58±0.51 (100%)
1.0	3.5±0.54 (100%)	**5.58±0.79 (100%)**
1.5	2.6±0.89 (83%)	5.08±0.79 (100%)
2.0	2.4±0.54 (83%)	4.66±0.77 (100%)
2.5	4.5±0.48 (83%)	4.6±0.54 (55%)

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

[#] *Concentrations in normal MSB_5 medium*

Results and Discussion

After 6 weeks of culture on induction medium, immature embryos produced watery and translucent callus. This callus could not regenerate but enclosed in it was a hard and compact region that produced shoot buds after first subculture.

Modification of $CuSO_4$ in the basal medium exerted profound influence on the morphogenetic responses of barley culture. Though the callus induced on various levels of $CuSO_4$ had similar appearance but showed different morphogenetic potential during later stages of culture. Callus was induced and shoot buds were produced in the absence of $CuSO_4$ [Fig 1 (i)] but they could not develop into plantlets even if regeneration medium had normal concentration of $CuSO_4$ [Fig 2(i)] indicating the essentiality of $CuSO_4$ during all the stages of culture for successful plant regeneration. On rest other treatments shoot buds were produced [Fig 1(ii & iii)] and plantlets were regenerated [Fig 2 (ii-iv)].

There was a gradual increase in the number of shoot buds formed per explant with an increase in the $CuSO_4$ concentration in the induction medium but the percentage of responding cultures increased drastically when $CuSO_4$ concentration in the induction medium was doubled (i.e., 0.2 µM) than that in the basal MSB_5 medium (i.e.,0.1 µM). Percentage of responding cultures further increased at 0.5 µM $CuSO_4$ in the induction medium. All the cultures produced shoot buds with 0.5 µM & 1µM $CuSO_4$ in the induction medium, though, the highest number of shoot buds were produced when primary cultures were subcultured on medium with 1µM $CuSO_4$ [Table 1].

Table 2.: Regeneration response of barley var. BL-2 cultures initiated, subcultured and regenerated on MSB_5 medium with different concentrations of $CuSO_4$.

Modified conc. of $CuSO_4$ (in µM)	**Shoots regenerated per explant Average ±S.D. (Percentage regeneration)**			
	Induction, subculture and regeneration medium with 0.1 µM[#] $CuSO_4$ (Control)	Induction medium with modified conc. but subculture & regeneration medium with 0.1 µM[#] $CuSO_4$	Both Induction and subculture medium with modified conc. & regeneration medium with 0.1 µM[#] $CuSO_4$	Induction, subculture as well as regeneration medium with modified conc. of $CuSO_4$
-	1.5±0.7 (40%)	-	-	-
0		0	0	0
0.2		2±0 (50%)	2.5±0.7 (60%)	2±0 (60%)
0.3		2.33±0.57 (60%)	2±0 (66%)	2.25±0.5 (80%)
0.4		2.5±1 (66%)	2±0 (66%)	2.5±0.57 (80%)
0.5		2.5±0.57 (66%)	3±0 (66%)	4.3±0.57(100%)
1.0		2.6±0.57 (75%)	**5.25±0.95(100%)**	4.66±0.57 (100%)
1.5		2.5±0.57 (66%)	3.8±0.4 (83%)	3.6±0.57 (100%)
2.0		2.33±0.57 (60%)	3.6±0.89 (71%)	3±0 (100%)
2.5		2.2±0.57 (60%)	3±1 (55%)	2.5±0.7 (50%)

Picloram concentration was 20.7 µM, 12.47 µM & 0.2 µM in callus induction, subculture & regeneration medium, respectively.

[#] Concentrations in normal MSB_5 medium

The relative ability of various culture to form shoot buds also correlated with the ability to regenerate green plants. For the various types of induction - subculture - regeneration medium combinations, the percentage of regenerable cultures as well as the number of plantlets regenerated per explant increased with an increase in the $CuSO_4$ concentration in the induction medium [Table 2]. For eachtype of Induction – subculture – regeneration medium combination, these values were maximum when the cultures were induced on 1 µM $CuSO_4$. Further increase in $CuSO_4$ concentration showed a constant decrease in the regeneration. On induction, subculture & regeneration medium with $CuSO_4$ concentration ranging from 0.5 µM to 2 µM not only 100% of the cultures formed shoot buds but also all of them

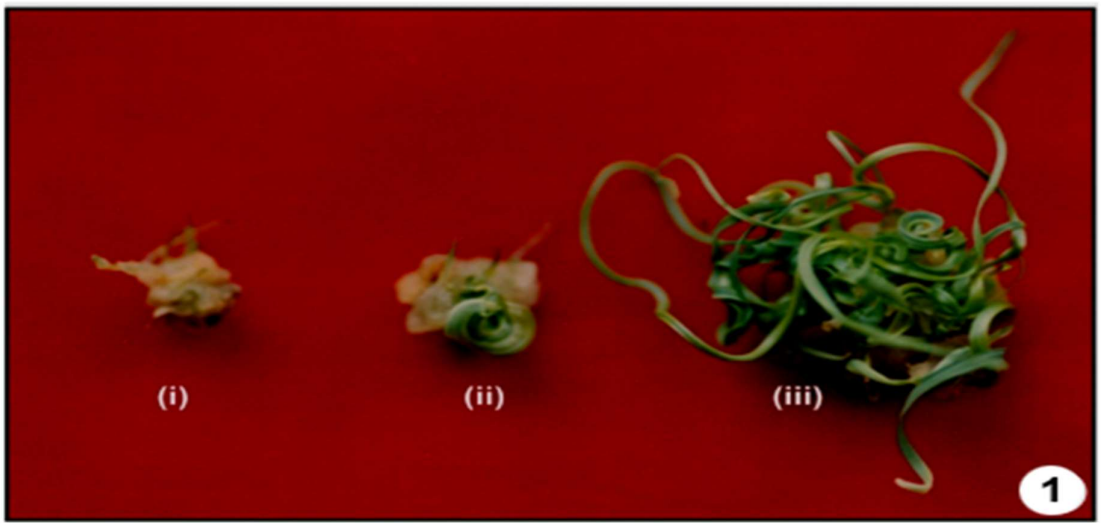

Figure 1. Shoot buds formed from cultures induced and subcultured on MSB_5 medium with picloram 20.7 µM & 12.47 µM respectively. *(i) Absence of $CuSO_4$ (ii) 0.1 µM $CuSO_4$ (iii) 1 µM $CuSO_4$*

Figure 2. Plant regeneration on normal regeneration medium [MSB_5 + picloram (0.2 µM)] from cultures induced and sub-cultured on MSB_5 medium with picloram (20.7 µM & 12.47 µM, respectively). *(i) Absence of $CuSO_4$ (ii) 0.1 µM $CuSO_4$ i.e., Control (iii) 1 µM $CuSO_4$ (iv) 2 µM $CuSO_4$*

regenerated plantlets. The maximum number of regenerated plantlets and the highest percentage of regeneration was observed when the $CuSO_4$ concentration in both the induction and subculture medium was 1 μM and 0.1 μM in the regeneration medium. At this combination 100% of the explants produced shoot buds and regenerated plantlets (c.f. 40% of control). The number of plantlets regenerated per explant was also 3.5 folds more than the control [Table 2].

Among the various micronutrients, $CuSO_4$ has gained utmost importance for its ability to enhance embryogenic callus growth, shoot bud differentiation and green plant regeneration. Murashige and Skoog[17]observed that copper added in the range from 0.03 μM to 30 μM was without effect on the growth of tobacco tissue though they recommended 0.1 μM $CuSO_4$ to be added in the basal medium. Later studies showed that the MS level of $CuSO_4$ was sub-optimal for various plant species. Callus induction as well as regeneration response of barley cultures was found to be dependent on the level of $CuSO_4$ incorporated in the basal medium. Variation in the morphogenic response of cultures with the change in $CuSO_4$ concentration has also been observed in other plants[19-23].

With the optimized concentration of $CuSO_4$ in the present study, 100% of the explants regenerated green shoots. Also, the number of plantlets regenerated per explant increased 3.5 folds. Our results are in agreement with the enhanced regeneration reported previously in rice, barley and other plant species by the addition of higher level of $CuSO_4$ in the basal medium. Dahleen discovered that surprisingly high levels of copper included in the culture medium facilitated regeneration of more green plants in barley[24]. Elevated copper concentrations increased the number of green plants per embryo from 1.9 to 2.4 fold and slightly increased the percentage of green plants in North American barley cultivars[25]. Regeneration upto 93.7% and more number of regenerated plants through increased level of $CuSO_4$ was achieved by Castillo *et al* in cultivars grown in Spain[12]. Beneficial effect of higher level of $CuSO_4$ was also observed by Zhang *et al* in barley[26]. We could achieve a higher regeneration frequency (100%) than all the above reports perhaps because we optimized the $CuSO_4$ level in the medium differently for the various stages of culture. Beneficial effect of copper on shoot regeneration has also been reported in wheat, triticale, rape, sorghum, tobacco, chili pepper, rose, *Withania* and cucumis[27-34].

The exact mechanism for this enhancement of regeneration is not known. It has been reported that copper plays an important role in several metabolic activities like protein and carbohydrate metabolism[35,36]. Copper is considered as an important constituent of several enzymes that might play a role in plant regeneration[28]. Numerous enzymes, including cytochrome oxidase, polyphenol oxidase, copper/zinc superoxide dismutase (Cu/Zn-SOD), amino oxidase, and phycocyanin require copper as a cofactor[37-39]. Copper ions participate in the electron transport chain[40-42] and cell wall metabolism[43] and act as cofactors that facilitate ethylene receptors to bind to ethylene[43,44]. Copper ions also protect cells against oxidative damage[45] and contribute to the production of hydroxyl[46] and to a wide range of biochemical pathways affecting DNA methylation[47,48]. Copper also inhibits endophytic bacterial contamination in cultures[49].

Though the higher level of copper enhanced regeneration but levels higher than the optimum showed decline in morphogenesis. Decline in growth due to higher doses of copper has also been reported in *Tinospora cordifolia*[50]. Lombardi and Sebastiani[51] reported toxic effect of higher copper levels on *Prunus* cultures. Metal ions such as Cu^{2+} are considered as essential trace elements may become toxic causing inhibition of growth and metabolism and even death of the organism[52]. Excess of Cu^{2+} may cause a

range of deleterious effects including inhibition of photosynthesis, pigment synthesis and damage to the plasma membrane permeability and other metabolic disturbances[53]. Copper ions can also act as a mediator of DNA sequence variation and therefore, their incorporation in the induction medium should be treated with caution[54].

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