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# INFLUENCE OF CALCIUM CHLORIDE AND IT'S OPTIMIZATION FOR HIGH FREQUENCY *IN VITRO* PLANT REGENERATION IN BARLEY (*HORDEUM VULGARE* L.) AND RICE (*ORYZA SATIVA* L.)

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The effect of calcium chloride on callus induction and plant regeneration from cultured immature embryos of Indian barley (*Hordeum vulgare* L. cultivar BL-2) and seeds of indica rice (*Oryza sativa* L. cultivar Pusa Basmati-1) has been investigated. Murashige and Skoog's medium containing Gamborg's medium vitamins and 20.7 µM, 12.47 µM & 0.2 µM picloram was used for callus induction, subculture and plant regeneration in barley cultures respectively. Rice callus was induced on Murashige and Skoog's medium supplemented with 11.31µM 2,4-dichlorophenoxyacetic acid and subsequently regenerated on MS medium containing 2.68 µM 1-naphthaleneacetic acid and 8.87 µM 6-benzylaminopurine. The basal media at different stages of culture were supplemented with different levels of calcium chloride (0 to 29.9 mM). Calcium chloride was found to be essential for plant regeneration. Higher level of calcium chloride at various phases of culture prior to regeneration was beneficial for regeneration of both barley and rice cultures. The optimum requirement was same for the two plants though for both the plants this level varied with the phases of culture. Regeneration frequency up to 100% with 2-2.5 folds higher number of regenerated plantlets was achieved when calcium chloride concentration in the induction and subculture media was raised to 14.95 mM and reduced to 2.99 mM in the regeneration medium.

**Key-words:** Calcium chloride, Callus induction, Plant regeneration, Barley, Indica rice

## Introduction

Barley and rice are important cereal crops grown mainly for food, feed, malting and brewing purposes that make them prime target for tissue culture and genetic manipulation. Rice was first, among the cereals, to be regenerated from callus[1]. Since then tissue culture has been applied to various cereals. Plants have been regenerated from different explants of barley and rice. These include mature and immature embryos[2-7], seeds[8-11], leaves[12,13], root-segments[14,15], young inflorescences[16,17], coleoptile[18,19] and seedling explants[20,21].

A prerequisite for the application of tissue culture technology for plant breeding and genetic transformation is the availability of efficient and reproducible plant regeneration protocol. The efficiency of this protocol is influenced by several factors including the composition of the basal medium, plant growth regulators and the genotype. A survey of published literature shows that MS is the most frequently used medium for culture of various plant species. The MS medium was especially aimed at optimization of mineral components of the medium for the growth of tobacco callus and may not be optimum for other types of cultures or groups of plants as various plant species

have their specific requirement for various nutrients. Numerous studies have investigated the relationship between mineral nutrients and explant growth[22-31]. Grusak[32], Ramage and Williams[33] reviewed the effect of mineral nutrients on plant morphogenesis.

Specific modifications of the basal medium have been reported to influence callus induction and plant regeneration in wheat[34-37], rice[38-49], barley[4,50-55,] sorghum[56,57], finger millet[58,59], rye[60]. Most of the studies concerning the role of mineral nutrients examined the effect of micro-nutrients and only few are related with macro-nutrients. Carman *et al.*[61] found that the medium containing double concentration of macro-nutrients of MS produced more embryogenic protuberances in wheat than any other medium tested. He *et al.*[62] could enhance embryoids formation by modifying the concentration of five macro-nutrients. Khanna and Raina[63], Saharawat and Chand[42] achieved better plant regeneration in rice with higher levels of $NH_4NO_3$ than in MS. In studies by Chauhan and Kothari[44,64] lower levels of $KH_2PO_4$ and $Fe_2(SO_4)_3$ proved favourable for callus induction and plant regeneration in rice. The present investigation was carried out to evaluate the potential of various levels of $CaCl_2$ for morphogenic callus induction and plant regeneration in barley and rice and to optimize it's concentration in the basal medium for the culture of these cereals.

**Materials and Methods**

Immature embryos of barley (*Hordeum vulgare* L. cultivar BL-2) and mature seeds of indica rice (*Oryza sativa* L. cultivar Pusa Basmati-1) were used as explant. Barley immature caryopses were procured from Agriculture Research Station, Durgapura, Jaipur, Rajasthan and surface sterilized by soaking for 30 sec in 70% (v/v) ethanol, three min in 0.1% aqueous solution of mercuric chloride 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. Mature rice caryopses were procured from GB Pant University of Agriculture and Technology, Pant Nagar, Uttaranchal, dehusked and surface sterilized with 0.1% aqueous solution of mercuric chloride for eight min followed by five rinses with sterile distilled water.

Protocol for the culture of barley immature embryos:

Immature embryos of barley were cultured on $MSB_5$ medium based on basal MS medium[65] with $B_5$ vitamins[66] supplemented with 20.7 μM picloram, different concentrations of $CaCl_2$ and 3.0% (w/v) sucrose. The leaves and roots of the germinating zygotic embryo were removed after 6 wks and the primary callus was subcultured. From each treatment one half of the primary calli were subcultured on medium with modified concentration of $CaCl_2$ and the other half on medium with normal concentration of $CaCl_2$. Subculture media were supplemented with 12.47 μM picloram. The calli after 4 wks on subculture media were transferred to $MSB_5$ medium supplemented with 0.2 μM picloram for regeneration. The regeneration medium for cultures on normal $MSB_5$ medium had normal concentration of $CaCl_2$ while the cultures subcultured on medium with modified concentration of $CaCl_2$ were regenerated both on normal $MSB_5$ regeneration medium and on medium with modified concentration of $CaCl_2$ as per the callus induction and subculture media. The regenerated shoots were rooted on $MSB_5$ medium supplemented with 2.85μM IAA and solidified with 0.2% (w/v) phytagel.

Protocol for the culture of rice immature seeds:

The MS medium supplemented with 11.31μM 2,4-D, different concentrations of $CaCl_2$ and 3.0% (w/v) sucrose served as basal medium for the culture of rice seeds. Primary calli were transferred to the regeneration media with 2.68 μM NAA and 8.87 μM BAP after 4 weeks without

any prior subculture. As for barley, rice cultures were regenerated on the normal MS regeneration medium and on regeneration medium with modified concentration of $CaCl_2$ as per the callus induction medium. Well-developed root system was formed on MS medium supplemented with 2.86 µM NAA and solidified with 0.2% (w/v) phytagel.

Thus the effect of various concentrations of $CaCl_2$ could be examined on various stages of culture in barley and rice. In the entire study the pH of media were adjusted to 5.8 and 0.8% bacteriological grade agar (Qualigens, India) was used as gelling agent before autoclaving at 121°C and 1.06 kg/cm$^2$ pressure for 15 min. The cultures were maintained at 26±1°C under a photoperiod of 16/8 hrs (light/dark) and at a light intensity of 25µmol min$^{-2}$sec$^{-1}$.

**Results and Discussion**

Barley immature embryos cultured on $MSB_5$ medium supplemented with picloram and various levels of $CaCl_2$ produced watery and translucent callus (Fig A) that enclosed a hard compact morphogenic region. Visible shoot buds could be observed after first subculture that elongated into shoots on regeneration medium. Primary callus in rice was creamish, compact and embryogenic. Initiation of callus was not dependent on $CaCl_2$ concentration added in the induction medium, however, it affected the development of later stages. The regeneration was influenced by the $CaCl_2$ concentration added in the induction as well as subculture and the regeneration media.

Callus was formed in rice and shoot buds were formed in barley (Fig B) on medium devoid of $CaCl_2$ but for regeneration the presence of $CaCl_2$ was found to be essential (Table 1,2&3). Studies on tobacco[29] and wheat[62] also revealed the fact that calcium is not essential for callus induction and shoot meristem initiation though it strongly affects the further development and differentiation potential of this callus. In these studies, calcium was only depleted from the medium by shoot forming tobacco cultures during post meristem formation i.e. growth phase. It has been proposed that in the absence of calcium, cultured plants may continue to grow for sometime utilizing endogenous calcium[67,68]. Lack of calcium caused a complete absence of the morphogenic process in *Eucalyptus urophylla* though small amount of calcium could be supplied from the agar[69]. It has been assumed by Fageria[70] and Marschner[71] that at very low availability of Ca (Ca deficiency) the plasma membranes of cells are adversely affected leading to ion leakage and unspecific uptake. Slightly increasing the Ca concentration in the nutrient solution then rapidly restores the membrane functionality, so that the uptake of other cations is enhanced and leakage reduced. Further increasing the Ca concentrations in the nutrient solution then turns the positive synergistic effect of the nutrients into an antagonistic cation competition for uptake. Calcium has been reported as an essential plant nutrient by White and Broadley[72]. Perhaps the morphogenic rice cultures required exogenous calcium for regeneration as the endogenous supply fell below a critical threshold during callus development in initial phase.

Higher concentrations of $CaCl_2$ were beneficial for shoot bud formation in barley and embryogenesis in rice. The rate of regeneration was also enhanced by higher levels of $CaCl_2$ than in MS medium (Fig C-H). In barley, the average numbers of shoot buds formed per explant almost doubled on medium with the increased concentration of $CaCl_2$ and remained almost constant for all the treatments except control. All the explants produced shoot buds when the primary callus induced on $MSB_5$ medium with higher concentrations of calcium chloride were subcultured on the normal $MSB_5$ medium. For cultures induced as well as subcultured on higher concentration of $CaCl_2$, the

number of shoot buds produced per explant remained almost constant but the percentage of responding cultures increased with an increase in the $CaCl_2$ concentration in the medium and was raised to 100% when the $CaCl_2$ level in the induction and subculture medium was raised to 14.95 mM. On contrary, though all the cultures induced on higher concentration of $CaCl_2$ and subcultured on normal produced shoot buds but only 50% of them regenerated (Table 2). Also less than half of the shoot buds of the regenerable cultures developed into plantlets. The cultures induced and subcultured on higher concentrations of $CaCl_2$ exhibited better regeneration (Fig C&D). Irrespective of the $CaCl_2$ concentration in the regeneration medium, for these cultures the percentage of regeneration and the number of regenerants per explant increased with an increase in the $CaCl_2$ concentration in the induction and subculture medium. Comparatively better regeneration of these cultures was observed on regeneration medium with normal concentration of $CaCl_2$. Rooting was normal in the regenerated plantlets (Fig I).

In rice also, a combination of modified induction-modified regeneration medium was poorer to the modified induction-normal regeneration medium (Table 3). With the normal concentration of $CaCl_2$ in the regeneration medium, the percentage of regenerable cultures as well as the average number of plantlets regenerated per explant gradually increased with an increase in $CaCl_2$ in the induction medium (Fig E&H).

Among all the concentrations of $CaCl_2$ tested, in both barley and rice, induction and subculture on 14.95 mM and regeneration on normal (2.99 μM) gave the optimum results where 100% of the explants produced morphogenic callus and regenerated plantlets (c.f. 35% of control culture in barley and 60% in rice). The number of plantlets regenerated per explant was also 2-2.5 folds more than the controls in the two plants. Enhancement of morphogenic potential of the cultures with increase in macro-nutrients concentration of the medium has also been reported by He *et al.*[62] and Jansen *et al.*[73]. He *et al*[62] observed an increase in the formation of white callus destined to form typical embryoids on higher concentration of $CaCl_2$. Jansen *et al.*[73] also reported two folds increase in the number of somatic embryos from *Daucus carota* suspension cultures due to an increase in calcium concentration in medium.

The present study also revealed that though barley and rice had different requirements for plant growth regulators and followed different pathways for morphogenesis but had same requirement of $CaCl_2$. According to Timmers *et al.*[74] $Ca^{2+}$ plays a role in the control of embryogenesis while Capitani and Altamura[75] assume that the ion is involved in the control of all types of meristem organization, not only the embryoids. Our results support the latter view as not only the embryogenesis in rice but also the organogenesis in barley was enhanced by the optimization of $Ca^{2+}$ concentration in the medium.

The exact mechanism for this enhancement is not known though an increase in calcium concentration has been reported to counteract, to a certain extent, inhibitory effect of 2,4-D on somatic embryogenesis[73] and activates the accumulation of flavonoids[76]. Elevated level of $Ca^{2+}$ increases the total protein and sugar content, peroxidase specific activity and changes the histological characteristics that are related to somatic embryogenesis, differentiation, morphogenesis and microplants growth[69,77].

Thus the present study concludes that the level of $CaCl_2$ in the MS medium is not optimum for barley and rice tissue culture. Furthermore, the two plants have an equal requirement for $CaCl_2$ though this requirement varies with the stage of culture in both the plants. Optimization of $CaCl_2$ for the various stages resulted in enhanced plant regeneration in barley and rice.

**Table 1 - Callus induction and shoot bud differentiation from immature embryos of barley *Hordeum vulgare* var. BL-2 in response to $MSB_5$ medium with various concentrations of $CaCl_2$**

| Concentration of $CaCl_2$ in callus induction medium (mM) | Concentration of $CaCl_2$ in callus subculture medium (mM) | Average number of shoot buds formed per explant after 10 weeks |
|---|---|---|
| 0 | 2.99[#] | 1.8 (40) |
| | 0 | 1.2 (25) |
| 2.99[#] | 2.99[#] | 2.33 (54) |
| 5.98 | 2.99[#] | 4 (100) |
| | 5.98 | 4.57 (58) |
| 8.97 | 2.99[#] | 4.16 (100) |
| | 8.97 | 4.5 (60) |
| 11.96 | 2.99[#] | 4.2 (100) |
| | 11.96 | 4.55 (75) |
| 14.95 | 2.99[#] | **4.4 (100)** |
| | 14.95 | 4 (100) |
| 29.9 | 2.99[#] | 3.5 (80) |
| | 29.9 | 3.2 (60) |

Picloram concentration was 20.7 µM in the induction and 12.47 µM in the subculture medium.

[#] Concentrations in normal $MSB_5$ medium

**Table 2 - Regeneration response of barley *Hordeum vulgare* l. var. BL-2 cultures initiated, subcultured and regenerated on $MSB_5$ medium with various concentrations of $CaCl_2$**

| Concentration of $CaCl_2$ in callus induction medium (mM) | Average number of shoots regenerated per explant (Percentage regeneration) | | |
|---|---|---|---|
| | $S_NR_N$ | $S_MR_N$ | $S_MR_M$ |
| 0 | 0 | 0 | 0 |
| 2.99[#] | 1.6 (35) | 1.6 (35) | 1.6 (35) |
| 5.98 | 2 (50) | 1.66 (50) | 2 (33) |
| 8.97 | 2 (50) | 2 (50) | 2.33 (50) |
| 11.96 | 2 (50) | 2.3 (75) | 3 (66) |
| 14.95 | 1.5 (50) | **3 (100)** | 3 (75) |
| 29.9 | 1 (40) | 2.2 (70) | 1.8 (50) |

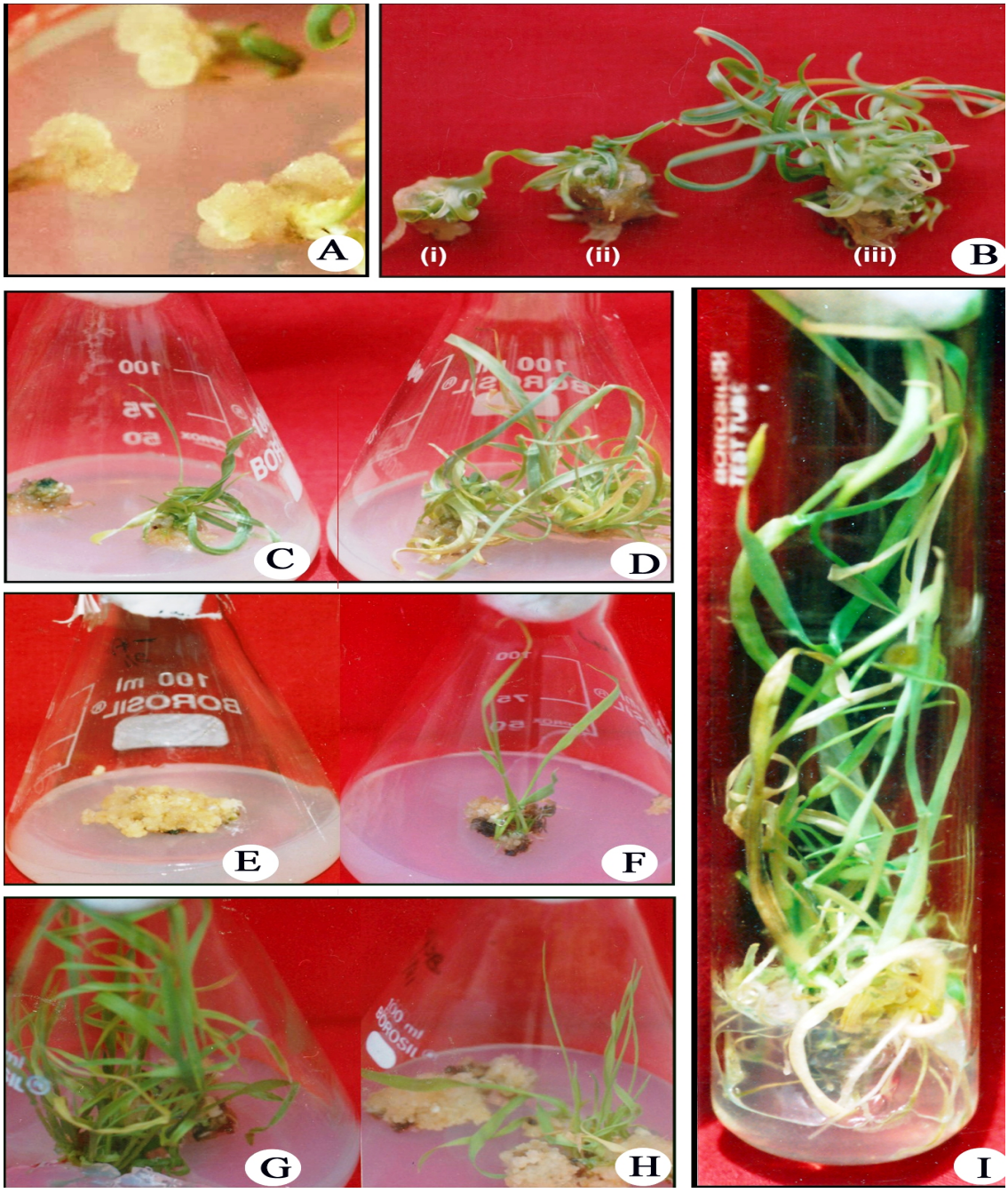
Picloram concentration was 20.7 µM in the induction, 12.47 µM in the subculture and 0.2µM in the regeneration medium.

[#] Concentrations in normal $MSB_5$ medium

$S_N$,$R_N$ : Subculture and regeneration medium respectively with normal (N) conc. of $CaCl_2$.

$S_M$,$R_N$ : Subculture medium with modified (M) and regeneration medium with normal (N) conc. of $CaCl_2$.

$S_M$,$R_M$ : Subculture and regeneration medium respectively with modified (M) conc. of $CaCl_2$.




**Effect of $CaCl_2$ on barley culture**

**A. Primary callus induced on $MSB_5$ + 20.7 µM pictogram**

**B. Shoot buds formed on subculture medium with**
**(i) 2.99 (ii) 8.97 (iii) 14.95 mM $CaCl_2$**

**C. Regeneration of plantlets in control cultures**

**D. Plantlets formation from cultures induced and subcultured on 14.95 mM $CaCl_2$ supplemented media but regenerated on normal $MSB_5$ Plantlet formation in rice on normal MS medium from callus induced on medium with**

**E. Absence of $CaCl_2$ F. 2.99 mM $CaCl_2$ G.14.95 mM $CaCl_2$ H. 29.99 mM $CaCl_2$ I.Rooting of barley plantlets**

**Table 3 - Callus induction and plant regeneration from seeds of rice *Oryza sativa* l. var. Pusa Basmati-1 in response to various concentrations of $CaCl_2$ added to callus induction medium* and plant regeneration medium****

| Concentration of $CaCl_2$ in callus induction medium (mM) | Average number of plantlets regenerated per explant (Percentage regeneration) | |
|---|---|---|
| | Normal Regeneration medium | Regeneration medium + $CaCl_2$ concentration as in callus induction medium |
| 0 | 0 | 0 |
| 1.49 | 8 (50) | 0 |
| 2.99# | 16 (60) | 16 (60) |
| 5.98 | 23.25 (66) | 15 (60) |
| 8.97 | 27.75 (80) | 14 (60) |
| 11.96 | 39 (80) | 16.5 (40) |
| 14.95 | **41 (100)** | 6 (20) |
| 29.9 | 22.5 (67) | 5 (17) |

* Callus induction medium: MS + 2,4-D (11.3 µM)

** Plant Regeneration medium: MS + NAA (2.68 µM) + BAP (8.87 µM)

\# Concentration in normal MS medium

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