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GENETICS

Study of in vitro selection and plant regeneration of Indica rice tolerant to iron

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Abstract

Iron tolerant callus lines of Indica rice Vars. Khandagiri and Jagannath has been achieved on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l Kinetin, 3.0 mg/l 2,4-D, 12 mM Fe. Somatic embryos derived from both tolerant non-tolerant calluses are germinated on MS medium supplemented with 1.0 mg/l 6-benzylaminopurine (BA), 0.25 mg/l 1-napthalen-eacetic acid (NAA) and 12 mM Fe within four weeks of subculture. The growth parameters like fresh and dry weight of callus, growth tolerance index, enzyme activity (Catalase and Peroxidase) and biochemical markers were used as indicators of iron tolerance. Measurements as early as two weeks after the beginning of the treatments did not yield consistent results. However, the growth tolerance index in the four weeks after the beginning of treatments yielded significant differences among non-tolerant and tolerant calluses. The enzyme activity is enhanced in the tolerant calluses than non-tolerant one. The *in vitro* raised plantlets derived from tolerant callus lines are grown *in vitro* in the presence of BA, NAA and 12 mM iron. The tolerant plantlets are established in the pots with iron-rich soil. This study will help to develop iron tolerant rice plants for breeding program.

Highlights

- Khandagiri and Jagannath callus lines showed tolerance to Fe and the plants are also growing normally in the iron rich soil.
- The enzyme activities were significantly higher in tolerant callus lines and pigment content was also more in tolerant callus compare with non-tolerant calluses.

Keywords: Enzyme activity, Fe- tolerance, regeneration, *In vitro* selection, Indica rice

Rice (*Oryza sativa* L.) (2n = 24) belonging to the family Poaceae and subfamily, Oryzoidea is the staple food for half of the world's population and occupies almost one-fifth of the total land area covered under cereals. In the Eastern part of India, Odisha has the distinction of possessing about 15,000 traditional rice varieties out of 50,000 found in the world (Kshirsagar *et al*. 2012). The cultivation and production of rice are declined due to abiotic and biotic stresses. Iron toxicity is one of the most important abiotic stresses limiting rice production in lowland systems (Dobermann and Fair-Hurst 2000). It has been reported a major constraint of lowland acidic soils, swamps, coastal swamps and irrigated lands of Asia and Africa. About 128 million hectares of the irrigated and rainfed lands of the world are under rice cultivation. Due to nutrient deficiency or toxicity, rice production has been reduced in about

100 million hectares of these lands (Becker and Asch, 2005; Asch *et al.* 2005). In India, total 11.7 million hectare of land is affected by iron toxicity. Iron toxicity interfere a range of nutrient disorders and deficiencies particularly potassium, phosphorus, calcium, magnesium and zinc in plant metabolism (Ottow *et al*. 1983; Pathirana *et al*. 2002; Roy and Mandal, 2005; Mehraban *et al*. 2008). *In vitro* culture technique is an alternative method for induction of iron tolerant plants for genetic improvement program. During last two decades, plant regeneration via organogenesis as well as somatic embryogenesis using various explants as well as from cells and protoplasts of rice are well documented (Yamada *et al*. 1986; Abdullah *et al*. 1986; Chen *et al*. 1985; Roy and Mandal, 2005). The ability of the targeted plant cells to regenerate into plantlet and subsequently develop into a mature plant is pre-requited for genetic improvement. This can happen only if the calluses are embryogenic i.e. the calluses are capable of forming embryos. In general, Indica rice has a low regeneration potential as compared to Japonica rice (Rueb *et al*. 1994; Gairi and Rashid, 2004). The development of efficient protocol on somatic embryogenesis is a prerequisite for crop improvement research and also valuable tools in understanding the basic plant biology. So far, there are scanty reports on *in vitro* selection and regeneration of the heavy metal tolerant plant. In the present investigation, we report on the selection of iron tolerant callus line as well as plant regeneration of Indica rice varieties 'Khandagiri' and 'Jagannath' .

Materials and Methods

Induction of embryogenic callus

Semi-mature seeds of *Oryza sativa* vars. Khandagiri and Jagannath were collected from germplasm centre of Orissa University of Agriculture and Technology, Bhubaneswar, Odisha. The seeds were washed with 2% (w/v) detergent solution (Teepol) for 30 min, further ringed with 70% (v/v) ethanol for 5 min, surface sterilized with 0.2% (w/v) aqueous solution of mercuric chloride for 30 min, followed by three rinses in sterile distilled water. Immature zygotic embryos were aseptically cultured on Murashige and Skoog (1962) basal medium supplemented with various concentrations of BA or Kn (0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/l), NAA or 2, 4-D (0, 0.5, 1.0, 1.5, 2.0, 3.0 mg/l) alone or in combination for callus induction and plant regeneration. The pH of the media was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to the addition of 0.8% (w/v) agar (Qualigen, India). Routinely, 15 ml of molten medium was dispensed into 25 x 150 mm glass test tubes (Borosil, India), capped with non-absorbent cotton plugs wrapped with one layer of cheesecloth. The cultures were sterilized at 121 ^{0}C and 104 kPa for 15 min.

Development of Fe- tolerant Callus Culture

Friable embryogenic calluses (500 ± 20 mg) was transferred to MS medium supplemented with different concentrations of kinetin and 2,4-D singly or in combinations with various concentration of Fe (1.5 mM normal Fe content in MS medium (Control), 4 , 8, 10, 12 and 14 mM) for selection of iron tolerant calli. Stock solution in the form of $FeSO_4.4H_2O$ were filter sterilized by 0.45 µm Millipore membrane filter. The concentration of the test metal was selected (i.e. LD_{50}, sub-lethal dosage: two concentrations below LD_{50}; the concentrations above LD_{50}) on the basis of preliminary experiments). The cultures were incubated under 16h photoperiod with light intensity of 55µmol $m^{-2}s^{-1}$ provided by cool, white fluorescent lamps (Phillips, India) at 25 ± 2^{0}C. Subculturing was made every four week intervals. The media were solidified with 0.8% (w/w) agar-agar. Morphological observations and callus growth measurements were determined at 4-week intervals. Pre-weighed culture vials containing 15 ml of culture medium were inoculated with similar quantities of callus, and the inoculated vials were re-weighed to obtain the initial fresh weight of the callus inoculum. The final weight minus initial weight of calluses in different treatments was used to calculate the percentage of callus growth against control. The cultures were incubated at 25±2^{0}C in cool, white fluorescent light (55µmol $m^{-2}s^{-1}$) under a 16h photoperiod for four weeks. The experiment had 15 cultures per treatment and repeated three times.

Determination of Dry Weight of Callus

Every four weeks, the callus samples of known fresh weight (200 mg) were dried to constant weight at 700 C in an oven. The relative growth rate (RGR) in terms of fresh mass of the callus growth was calculated with the formula of Shah *et al.* (1993).

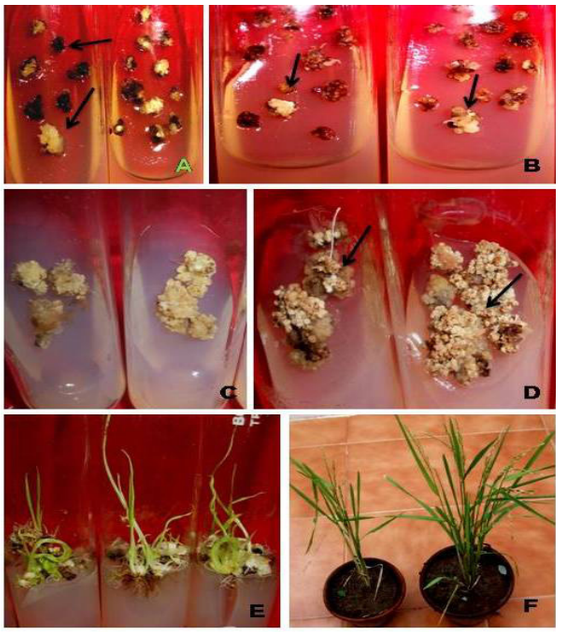

Figure 1

A &B. Embryogenic callus developed from immature zygotic embryos of Var. Khandagiri (A) and Var. Jagannath (B) on MS medium supplemented with 1.0 Kn + 3.0 mg/l 2,4-D + 12 mM Fe after four weeks of subculture.

C &D. Proliferation of embryogenic calluses (C) and germination Germination of somatic embryos on MS medium supplemented with 1.0 mg/l BA + 0.25 mg/l NAA + 12 mM Fe + 2% surose after three weeks of subculture.

E . Germination of somatic embryos of Var. Jaganath on 1.0 mg/l BA + 0.25 mg/l NAA + 12 mM Fe + 2% surose after three weeks of culture.

F. The tolerant regenerated plantlets establish in pot with panicle formation after two months of transfer

RGR = Ln (final mass) – Ln (initial mass)/ weeks

Growth Tolerance Index (GTI) was calculated using the formula:

GTI (%) = Mean% of callus growth in media with addition of Fe / Mean% of callus growth in media without addition of Fe x 100

Germination of Somatic Embryos

The group of both non-tolerant and tolerant embryogenic calluses was further transferred to ½ strength MS medium supplemented without or with 0.25 - 0.5 mg/l NAA, 0.25 – 1.0 mg/l BA, 12 mM Fe and 3% (w/v) sucrose for maturation and germination. The cultures were routinely maintained at every 4-week intervals. In another experiment, the embryogenic cultures were kept in the dark for two weeks that were then transferred to the light (16h Photoperiod) for germination of somatic embryos. Each treatment consisted of 15 replications, and the experiments were repeated two times.

Table 1. Effect of Kinetin, BA, NAA and 2,4-D on callus induction from immature zygotic embryo of *O.sativa* Var. Khandagiri and Var. Jagannath after four weeks of culture.

MS + growth regulators (mg/l)				Percent of explants forming callus (Mean ± SE)*	
Kn	BA	NAA	2,4-D	Var. Khandagiri	Var. Jagannath
0	0	0	0	0	0
0.5	0	0	0	0	0
1.0	0	0	0	22.4 ± 0.3 a	24.6 ± 0.7 b
1.5	0	0	0	26.6 ± 0.8 d	32.2 ± 0.8 d, e
0	0.5	0	0	0	0
0	1.0	0	0	12.8 ± 0.4 a	16.2 ± 0.6 a
0	1.5	0	0	18.4 ± 0.5 b	16.8 ± 0.7 a
1.0	0	0	2.0	54.6 ± 0.7 h	52.2 ± 0.6 f
1.0	0	0	3.0	74.8 ± 0.8 k	68.8 ± 0.6 h
1.5	0	0	3.0	72.6 ± 1.2 j	64.6 ± 0.8 g
1.5	0	0	4.0	62.4 ± 0.7 i	52.8 ± 0.7 f
0	1.0	0	3.0	26.4 ± 0.8 d	32.2 ± 0.4 d, e
0	1.5	0	4.0	30.4 ± 0.6 e	30.4 ± 0.6 d
1.0	0	2.0	0	26.8 ± 0.4 d	24.8 ± 0.5 b
1.5	0	3.0	0	30.8 ± 0.8 e	28.6 ± 0.7 c
2.0	0	4.0	0	32.2 ± 0.6 e, f	30.2 ± 0.6 d
0	1.0	2.0	0	24.6 ± 0.7 c	26.2 ± 0.8 b, c
0	1.5	3.0	0	28.4 ± 0.8 d, e	30.6 ± 0.7 d
0	2.0	3.0	0	32.3 ± 0.5 e, f	32.2 ±0.6 d, e
0	2.0	4.0	0	35.6 ± 0.4 g	34.2 ± 0.8 e

*15 replicate per treatment; repeated three times.

[a-k] Mean having the same letter in a column were not significantly different by Post-hoc multiple comparison tests P< 0.05.

Table. 2. Effect of different concentrations of Fe on the growth of callus of *O.sativa* Var. Khandagiri and Jagannath cultured on MS medium supplemented with 1.0 mg/l Kn, 3.0 mg/l 2,4-D and 3% sucrose after four weeks of culture.

Fe Concentration (mM)	Growth Tolerance Index (Mean ± SE)*	
	Var. Khandagiri	Var. Jagannath
1.5 (Control)	74.5 ± 1.6 f	68.8 ± 1.4 e
4.0 mM	68.6 ± 1.8 e	60.2 ± 1.2 d
8.0 mM	62.4 ± 1.2 d	59.4 ± 1.3 d
10.0 mM	56.8 ± 0.9 c	52.2 ± 1.1c
12.0 mM	50.6 ± 0.8 b	48.6 ± 1.5 b
14.0 mM	24.4 ± 0.7 a	± 1.2 a

*Data in parentheses indicate the percentage of reduction (-) / increase (+) relative to control. 15 replicate per treatment; repeated three times.

[a-f] Means having the same letter in a column were not significantly different by the post-hoc multiple comparison tests, P<0.05.

Table 3. Chlorophyll content (mg/g fresh weight basis) of tolerant and non-tolerant callus of *O.sativa* Var. Khandagiri and Var. Jagannath cultured on MS medium supplemented with 1.0 mg/l Kn, 3.0 mg/l 2,4-D and different concentrations of Fe after four weeks of culture.

Metal concentration (mM)	Chlorophyll content (mg/g fresh weight) of callus (Mean ± SE)*		
	Chloro-phyll-a	Chloro-phyll-b	Total chlorophyll (a + b)
Var. Khandagiri (Non-tolerant callus)			
1.5 (Control)	2.21 ± 0.5	1.72 ± 0.4	3.93 ± 0.9 a
4.0	2.87 ± 0.7 (+ 29.7)	1.89 ± 0.6 (+9.90)	4.76 ± 1.3 d (+ 21.1)
8.0	3.12 ± 0.4 (+ 41.2)	2.22 ± 0.7 (+29.0)	5.34 ± 1.1 f (+35.9)
10.0	3.25 ± 0.5 (+ 47.0)	2.36 ± 0.4 (+37.2)	5.61 ± 0.9 h (+42.7)
12.0	4.16 ± 0.7 (+88.2)	2.78 ± 0.3 (+61.6)	6.94 ± 1.0 k (+76.6)
14.0	4.86 ± 0.6 (+119.9)	3.14 ± 0.6 (+82.5)	8.00 ± 1.2 p (+103.6)
Var. Khandagiri (Tolerant callus)			
1.5 (Control)	2.62 ± 0.8	1.96 ± 0.3	4.58 ± 1.1 c
4.0	2.87 ± 0.7 (+9.54)	2.16 ± 0.6 (+10.2)	5.03 ± 1.3 e (+9.82)
8.0	3.15 ± 0.6 (+20.2)	2.62 ± 0.8 (+33.7)	5.77 ± 1.4 i (+25.9)
10.0	3.89 ± 0.8 (+48.5)	2.78 ± 0.2 (+41.8)	6.67 ± 1.0 j (+45.6)
12.0	4.16 ± 0.4 (+58.8)	3.56 ± 0.5 (+81.6)	7.72 ± 0.9 n (+68.5)
14.0	5.24 ± 0.5 (+100.0)	3.72 ± 0.6 (+89.8)	8.96 ± 1.1 q (+95.6)
Var. Jagannath (Non-tolerant callus)			
1.5 (Control)	2.06 ± 0.6	1.80 ± 0.5	3.91 ± 1.1 a
4.0	2.45 ± 0.4 (+18.9)	1.85 ± 0.6 (+ 2.77)	4.37 ± 1.0 b (+11.4)
8.0	3.12 ± 0.3 (+51.4)	2.34 ± 0.7 (+30.0)	5.46 ± 1.0 g (+41.5)
10.0	3.45 ± 0.7 (+67.5)	2.51 ± 0.8 (+39.4)	5.96 ± 1.5 k (+54.4)
12.0	4.14 ± 0.8 (+100.9)	3.28 ± 0.5 (+82.2)	7.42 ± 1.3 m (+92.2)
14.0	4.26 ± 0.7 (+106.8)	3.68 ± 0.4 9101.6)	7.94 ± 1.1 o (+105.7)
Var. Jagannath (Tolerant callus)			
1.5 (Control)	2.08 ± 0.4	2.13 ± 0.5	4.21 ± 0.9 b
4.0	2.45 ± 0.5 (+17.8)	2.22 ± 0.6 (+4.22)	4.67 ± 1.1 d (+10.92)
8.0	2.67 ± 0.6 (+28.4)	2.89 ± 0.7 (+35.7)	5.56 ± 1.3 h (+32.06)
10.0	3.82 ± 0.7 (+83.6)	3.24 ± 0.4 (+52.1)	7.06 ± 1.1 l (+67.7)
12.0	4.53 ± 0.4 (+117.8)	3.58 ± 0.6 (+68.0)	8.11 ± 1.0 p (+92.6)
14.0	4.92 ± 0.5 (+136.5)	3.94 ± 0.5 (+84.9)	8.86 ± 1.0 q (+110.4)

*Four replicates per treatment; repeated three times.
Data in parenthesis indicate the percentage of reduction (-)/ increase (+) relative to control.
[a – q] Means having the same letter within a column were not significantly different by the post-hoc multiple comparison tests, p< 0.05.

Table 4. Total protein content (µg/g fresh weight basis) of tolerant and non-tolerant callus of *O.sativa* Var. Khandagiri and Var. Jagannath cultured on MS medium supplemented with 1.0 mg/l Kinetin and 3.0 mg/l 2,4-D and different concentrations of Fe after four weeks of culture.

Metal concentration (mM)	Source of callus	
	Tolerant callus	Non-tolerant callus
Var. Khandagiri		
1.5 (Control)	2.79 ± 0.8 a	2.90 ± 0.5 c
4.0	3.24 ± 0.6 b (+16.1)	2.96 ± 0.4 c (+2.0)
8.0	3.88 ± 0.7 d (+39.0)	3.11 ± 0.7 d (+21.0)
10.0	4.42 ± 0.6 e (+58.4)	3.32 ± 0.8 e (+14.5)
12.0	4.68 ± 0.8 f (+67.7)	3.84 ± 0.4 f (+32.4)
14.0	5.12 ± 0.5 h (+83.5)	4.05 ± 0.5 g (+39.6)
Var. Jagannath		
1.5 (Control)	2.82 ± 0.5 a	2.12 ± 0.5 a
4.0	3.56 ± 0.6 c (+26.2)	2.25 ± 0.6 a (+6.13)
8.0	3.92 ± 0.7 d (+39.0)	2.58 ± 0.6 b (+21.7)
10.0	4.77 ± 0.5 g (+69.1)	2.96 ± 0.8 c (+39.6)
12.0	5.23 ± 0.6 h (+85.5)	3.01 ± 0.9 d (+41.9)
	5.66 ± 0.4 i (+100.7)	3.24 ± 0.7 e (+52.8)

*Data in parenthesis indicate the percentage of reduction (-)/ increase (+) relative to control. Four replicates/ treatment; repeated thrice.

$^{a-i}$ Means having the same letter within a column were not significantly different by the post-hoc multiple comparison tests, $p<0.05$ level.

Table. 5. Catalase activity of toloerant and non-tolerant callus of *O.sativa* Var. Khandagiri and Var. Jagannath grown on MS medium supplemented with 1.0 mg/l Kinetin and 3.0 mg/l 2,4-D and different concentrations of Fe after four weeks of culture.

Variety	Catalase activity (µmol H_2O_2 destroyed/ min/mg protein)	
	Tolerant	Non-tolerant
Var. Khandagiri		
1.5 (Control)	24.2 ± 1.2	23.6 ± 0.9
4.0	28.4 ± 1.0 (+17.3)	26.4 ± 0.8 (+11.9)
8.0	32.4 ± 1.2 (+33.9)	28.6 ± 0.7 (+21.2)
10.0	40.8 ± 1.0 (+68.6)	32.8 ± 0.6 (+38.9)
12.0	45.2 ± 1.3 (+86.8)	24.8 ± 0.9 (+5.08)
14.0	53.6 ± 1.1 (+121.5)	19.56 ± 0.8 (-17.1)
Var. Jagannath		
1.5 (Control)	21.8 ± 1.0	22.2 ± 0.7
4.0	25.6 ± 1.2 (+17.4)	26.6 ± 0.9 (+19.8)
8.0	28.4 ± 1.4 (+30.3)	29.6 ± 0.8 (+33.3)
10.0	30.6 ± 1.2 (+40.4)	24.2 ± 0.7 (+9.00)
12.0	36.8 ± 1.3 (+68.8)	11.4 ± 0.9 (- 17.11)
14.0	42.8 ± 1.4 (+96.3)	8.34 ± 0.6 (- 48.9)

*Data represent in parenthesis indicate the percentage of reduction (-) / increase (+) relative to control. Four replicates per treatment; repeated three times.

Table. 6. Peroxidase activity of toloerant and non-tolerant callus of *O.sativa* Var. Khandagiri and Var. Jagannath grown on MS medium supplemented with 1.0 mg/l Kinetin and 3.0 mg/l 2,4-D and different concentrations of Fe after four weeks of culture.

Variety	Peroxidase activity (µmol H_2O_2 destroyed/min/mg protein)	
	Tolerant	Non-tolerant
Var. Khandagiri		
1.5 (Control)	12.6 ± 0.8	11.8 ± 0.8
4.0 mM	16.2 ± 1.1 (+28.6)	14.6 ± 0.7 (+23.7)
8.0 mM	23.4 ± 0.9 (+85.7)	18.2 ± 0.6 (+54.2)
10.0 mM	28.8 ± 0.8 (+128.6)	22.4 ± 0.5 (+72.9)
12.0 mM	32.6 ± 0.7 (+158.7)	17.8 ± 0.7 (+50.8)
14.0 mM	42.8 ± 0.8 (+239.7)	10.1 ± 0.6 (- 14.4)
Var. Jagannath		
1.5 (Control)	14.2 ± 0.8	14.6 ± 0.6
4.0 mM	20.6 ± 0.6 (+45.07)	18.4 ± 0.5 (+26.0)
8.0 mM	25.6 ± 0.7 (+80.3)	22.5 ± 0.7 (+54.1)
10.0 mM	30.2 ± 0.4 (+112.7)	26.2 ± 0.6 (+65.7)
12.0 mM	35.8 ± 0.6 (+163.4)	18.4 ± 0.8 (+27.4)
14.0 mM	46.4 ± 0.9 (+226.8)	12.8 ± 0.9 (- 12.3)

*Data represent in parenthesis indicate the percentage of reduction (-) / increase (+) relative to control. Four replicates per treatment; repeated three times.

Acclimatization of plantlets

The somatic embryo-derived tolerant plantlets were transferred to the soil mixture at the ratio of 1: 2: 1 (Sand: Iron-rich Soil: Cow dung) and kept in the greenhouse with 85% relative humidity. The nutrient medium with 12 mM Fe and without organic is given to the plants in every two-day intervals. About 70% plantlets survived in the green house and flowered after two months of the transfer.

Chlorophyll and protein estimation

Callus samples (500 mg fresh weight basis) from each tolerant and non-tolerant source are collected at 4 week intervals for the estimation of chlorophyll. The callus was homogenized with 80% acetone in the dark. The amount of chlorophyll is estimated according to Vernon (1960). Pigment content was expressed as mg/gm fresh weight of the sample. For the analysis of protein content, fresh weight samples (100 mg) were analyzed by conventional micro-Jeldahl method for the estimation of total nitrogen. Soluble nitrogen was determined by this method after precipitating the protein in the extract of the fresh material with trichloroacetic acid (Anonymous 1970).

Enzyme Extraction and Assay

Peroxidase

Fresh callus samples (500 mg) from each treatment were collected at two weeks intervals, homogenized with mortar and pestle in cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble polyvinylpyrrolidone and 15 mg sodium ascorbate. The homogenate was filtered through four layers of Micacloth and centrifuged at 12,000 x g for 10 min at 4^0 C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1M phosphate buffer (pH 6.1), 4mM guaiacol, 3mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance at 420 nm was measured using a UV spectrophotometer. The levels of enzyme activities are expressed as μmol H_2O_2 destroyed/min/mg.protein.

Catalase

Fresh Callus sample (500mg) from each treatment were collected at 4-week intervals, homogenized in 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 1000 x g for 10 min at 4^0C. One milliliter of supernatant was added to the reaction mixture containing 1 ml 0.1 M H_2O_2 and 3 ml 0.1 M sodium phosphate buffer (pH 7.0). The reaction was stopped by adding 1.0 ml 2% H_2SO_4 after 1 min incubation at 20^0C. The acidified reaction mixture with or without the supernatant was titrated against 0.01 M $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The catalase activity isexpressed as μmol H_2O_2 destroyed/min/mg.protein.

Statistical analysis

The data pertaining to mean percentage of explants with calluses/treatment, regeneration frequency, chlorophyll content, total protein content and catalse and peroxidase activity of metal tolerant and non-tolerant sources were statistically analysed by analysis of variance. Between the treatments, the average figures followed by the same letter within a column in the tables were not significantly different at the $P < 0.05$ level (Post-Hoc Multiple Comparison Tests).

Results and Discussion

Embryogenic Callus induction

The present study indicates the establishment of the efficient protocol on the plant regeneration system through somatic embryogenesis of rice genotypes tolerance to iron. Both cytokinins and auxins were used for development of embryogenic callus. Among the two cytokinins and auxins tested, kinetin alone or kinetin in combination with 2,4-D showed the positive effect on callus proliferation from immature zygotic embryos. The effect of growth regulators on embryogenic calli development is presented in Table. 1. Initially, small yellowish white friable calli developed from immature zygotic embryos within four weeks of culture on MS medium supplemented with 0.5–1.5 mg/l Kn and 2.0–4.0 mg/l 2,4-D (Figure 1

A and B). The maximum proliferation of embryogenic calluses is noted in the medium containing 1.0 mg/l Kn supplemented with 3.0 mg/l 2,4-D. BA is not as effective in embryogenic callus induction as compared with Kn. The medium having Kn with 2,4-D was observed to be best for callus proliferation. The proliferated primary globular calluses are subsequently subcultured into similar medium for further proliferation of embryogenic calli. The medium devoid of growth regulators did not help in the proliferation of callus development. The maximum rate of embryogenic callus proliferation was noted in MS medium supplemented with 0.5–1.0 mg/l Kn, 3.0 mg/l 2,4-D in *O.sativa* Vars. Khandagiri and Jagannath. The proliferation of friable embryogenic callus in terms of fresh weight was maximum in the medium having Kn + 2,4-D as compared to Kn or BA + NAA and showed yellowish white coloration. Similar responses were observed when 2,4-D is replaced with NAA. The embryogenic calli development in somatic cells is often accompanied cellular stress. Moreover, 2,4-D, the most frequently used for induction of somatic embryogenesis, was known to activate many stress related genes supporting the hypothesis that somatic embryogenesis resulted due to extreme stress response of cultured cells. In some cases, however, induction medium favours both callus induction and stimulation of embryogenic response. The ability to form embryogenic callus is genotype dependent. Out of two genotypes tested, one genotype favored maximum embryogenic frequency as compared to another genotype.

Selection of Fe tolerant Embryogenic Callus

Embryogenic calli grown on medium containing MS basal salts supplemented with 1.0 mg/l Kn and 3.0 mg/l 2,4-D were transferred to subculture medium having similar composition but supplemented with different concentrations of Fe (1.5 mM (Control), 4, 8, 10, 12 and 14 mM). The callus rapidly proliferated into profuse yellowish white structures in 4 weeks on medium containing 4 and 8mM. Few colonies grew at higher concentrations of 10 and 12 mM Fe and other cultures did not show any sign of growth and turned brown within one week of culture. Subsequently, the calluses cultured on 10 and 12 mM grew rapidly into yellowish-white coloration (Figure 1C). The cultures that showed growth were separated and subcultured on respective medium at four week intervals. The tolerant calluses are again transferred onto a similar medium with 12 mM Fe or control medium. Significant growth is achieved on medium containing 12 mM Fe (tolerant) as compared with the control medium (non-tolerant). The GTI of non-tolerant and tolerant callus showed significant variation on medium containing different concentrations of Fe (Table 2). The tolerant calluses produced significantly more fresh and dry biomass at 12 mM Fe than the non-tolerant cultures. The growth of non-tolerant calluses is, however, stimulated by the low concentration of Fe. With further increases in Fe concentration, the callus showed a reduction in fresh weight by 40% at 12 mM Fe. The growth is arrested due to the high concentration of Fe in the growth media, which may be binding protein (Steffens,1990). The tolerant and non-tolerant calluses are maintained for a prolonged period on similar medium.

Pigment and Protein Analysis

Both tolerant and non-tolerant calluses are taken for biochemical analysis. The callus derived from tolerant lines showed the higher chlorophyll content as compared with non-tolerant lines (Table 3). Similar observations were made earlier on chlorophyll synthesis in tobacco callus due to the presence of the high concentration of manganese (Petolino and Collins 1985; Clairmont *et al.* 1986). The cholophyll a and b content in the tolerant sources increased 58.77 to 117.8% for chlorophyll a and 68.07 to 81.63% for chlorophyll b in the presence of 12 mM Fe (Table 3). The protein content is more in tolerant calli as compared with non-tolerant calli (Table 4). Mandal *et al.* (2004) reported that the Fe-tolerant rice cultivars absorb less Fe from root to leaves, indicating the governance of a physiological avoidance mechanism at any concentration of Fe in the leaves. Fe-tolerant

cultivars display superior performance under Fe-toxic condition partly due to Fe tolerance.

Enzyme activity

The enzyme activity was examined in both tolerant and non-tolerant calluses. The enzyme activities are enhanced for plant metabolism under stress condition and, therefore, may play a role in metal tolerance (Van Assche and Clijsters 1990). During the growth period, the activities of both catalase and peroxidase are significantly higher in tolerant calluses grown in the medium having 12 mM Fe in comparison with non-tolerant calluses (Tables 5 and 6). Higher activity of catalase and peroxidase indicated that the plants derived from tolerant calluses were under oxidative stress, a feature often associated with metal tolerance. Rout *et al.* (1999) reported that the both catalase and peroxidase activity were higher in tolerant calli of Brassica juncea and Brassica campestris grown on 0.8mM manganese and 0.24mM zinc containing growth medium. The catalase and peroxidase activity were generally high in crops grown in heavy metal polluted soil as reported earlier (Nashikkar and Chakrabarti,1994). Enzymatic oxidation of Fe was found to be the principal mechanism for higher oxidation activity of rice roots and chiefly responsible for root oxidation in addition to catalase and peroxidase enzymes (Mandal *et al.* 2004).

Germination of Somatic Embryos

The globular somatic embryos developed into plantlets on MS medium supplemented with 0.25 – 1.0 mg/l BA and 0.25 – 0.5 mg/l NAA within four weeks of culture. The medium devoid of growth regulators did not promote germination. Most of the somatic embryos were loosely attached to form an aggregate of embryos that could be easily separated; some somatic embryos arose from the base of other embryos to form clusters indicating secondary somatic embryogenesis. A high percentage of embryos germinated when 1.0 mg/l BA, 0.25 mg/l NAA and 12mM Fe were added to the medium. Within three weeks of transfer to maturation medium, about 90% of the somatic embryos germinated and developed roots without showing secondary callusing (Figure 1D). Rooted plantlets were transferred to pots containing a pot comprised of cow dung, sand and iron-rich soil in the ratio of 1:1;1 (v/v), respectively, and kept in the greenhouse for acclimatization (Figure 1E). After one month of transfer to the soil mixture, about 70% of the plantlets survived in the greenhouse. The plants are bearing panicles after 60 days of transfer to the pot (Figure 1F).

In Conclusion, the tolerant callus lines showed tolerance to Fe and the plants are also growing normally in the iron rich soil. The enzyme activities were significantly higher in tolerant callus lines and pigment content was also more in tolerant callus compare with non-tolerant calluses. Development of metal tolerant cell lines may be useful in crop improvement programmes. Further studies are necessary to identify the molecular mechanism in rice.

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