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In Vitro Selection and Plant Regeneration of $Cacl_2$ & Mn-Tolerant Plants from Leaf Callus of *Nicotiana Tabacum L.*

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Abstract: *The Nicotiana tabacum L. belongs to Solanaceae or night shade family is one of the most important economic crops, which has been considerate to fruit fly of plant kingdom because it becomes to a classical mode plant that could be cultured in vitro and gain regenerated transformation plant easily. In this experiment best swelling of explants and shoot bud obtain at the media concentration 1 mg/l NAA and 1 mg/l BAP with different combination of Mn & $CaCl_2$. and 0.5 mg/l IAA & 0.5mg/l BAP. The formation of callus induction was recorded (40%-100%) at higher concentration of 2, 4- D (2.0 mg/l) with different concentration of $CaCl_2$ and Mn. The average shoot length was maximum in the 1.0 mg/l BAP, 0.25 mg/l IAA, 0.5 mg/l GA_3 and least in the $CaCl_2$: $2H_2O$; 2mM + $MnS0_4$:H_2O; 2mM. The regenerated tolerant shoots rapidly multiplied on similar medium. Regenerated shoots (both tolerant and non tolerant) were cultured to half strength MS basal medium supplemented with 0.1 mg/l IAA and different combination of $CaCl_2$ & Mn for induction of rooting. After one week, about 92% of tolerant shoots were rooted with good root system. Well rooted explants transfer into the green house for the adaptation of environment.*

Keywords: *Ca, Mn, Tolerant, Phytohormones, Micropropagation.*

1. Introduction

Metals are important contaminants in the environment. Their presence has increased from human activities such as industry, agriculture and mining. Metals like Ni, Cu, Zn, Cd, Pb and Mn have various phytotoxic effects, including reduction of growth, photosynthesis, chlorophyll content, inhibition of enzyme activities and damage to chloroplasts and mitochondria [Reddy & Prasad, 1990]. However, genetic and physiological tolerance or adaptation processes have been shown to occur in numerous species and populations [Verkleij, et al., 1991].

Manganese is an essential trace element for plants and is present as a micronutrient in all media used for in vitro plant culture. The metal has a vital role in green plants for respiration and photosyntetic O_2 evolution, where it is required for the Hill reaction and electron transport. Manganese is a metal component of one of the isoforms of SOD, which has a key function for the protection of the photosynthetic apparatus from the harmful effects of O_2 activation. Moreover, Mn activates several enzymes such as NADPH decarboxylating malate dehydrogenase, malic enzyme, isocitrate dehydrogenese and nitrate reductase [Mukhopadhay and Sharma, 1991]. Excess Mn is one of the more important growth limiting factors in acid soils. Root injuries [Foy, 1984], shoot growth retardation [Kang and Fox, 1980], foliar chlorosis, necrotic spotting [Petolino and Collins, 1985], inhibition of chlorophyll synthesis and declines in the photosynthetic rate [Macfie and Taylor, 1992] are all symptoms of Mn toxicity. The critical toxicity levels vary widely across plant species or genotypes and environmental conditions [Wang et al., 1994]. Negative effects have also been reported at nuclear and chromosomal levels [Fiskesjo, 1988]. Manganese tolerant plants can be obtained through a traditional genetic approach, but this requires a large screening work of a great number of plants.

A previous research established an in vitro procedure for the selection of *Nicotiana tabacum var.* BEL W3 callus lines and regenerated plants tolerant to high doses of Mn (2 and 5 mM). The performance of the Mn-tolerant plants with regard to several morphological, anatomical and cytological characteristics, in comparison to the Mn-susceptible regenerated plants, was described [Santandrea, 1998]. In particular, Mn was also shown to affect the number of xylem elements and the degree of signification, which differed from plants grown in the presence of 2 and 5 mM Mn compared to the control. Moreover, in these plants, damaged chloroplasts and a reduction in their number were also observed, especially for those treated with 5 mM Mn.

With an aim of better explaining these previous results and to characterize Mn-tolerant plants from the physiological point of view, the present study was planned to development of inorganic compound tolerant plants of *Nicotiana tabacum* through *in vitro* culture method. To achieve

this goal different concentration of inorganic compounds ($CaCl_2$ & Mn) were used alone or in combination with one another.

2. Materials and Methods

Experimental studies were conducted on *N. Tabacum* collected from poly-house of Tectona Biotech Resource Centre (TBRC), Bhubaneshwar and brought to the laboratory. The elite plants selected were maintained under hygienic conditions. The explants were collected from three months old plant and also remove the mature leaves. For standardization of protocol for mass multiplication of tabacum species, the explants like leaf discs (diameter 8mM) were cut from the youngest fully expanded leaves were used as explants source.

2.1 Effects of Mn and $CaCl_2$ on callus induction and shoot regeneration of N. Tabacum:

Leafs of N. tabacum var. were surface sterilized with 0.1% $HgCl_2$ (7% active chlorine) for 20 min, then washed with sterile water three times then were inoculated into culture bottles containing 50ml of Murashige and Skoog's (MS) medium [Murashige & Skoog, 1962] with growth hormone BAP and NAA used with different concentration of inorganic compounds ($CaCl_2$ & Mn) inside the laminar air flow. The culture bottles were maintained at 25±2^{o}c continuous light (3000 lux) with a photoperiod of 16 hours daily followed by 8 hours dark period and 60-70% humidity. After the inoculation of Tobacco weekly observation was taken to examine their growth rate, changes in medium color, phenolic accumulation, checking of contamination in explants etc. Inoculated explants multiply in the media 1 mg/L NAA and 1 mg/L BAP with different combination of Mn & $CaCl_2$ and 0.5 mg/l IAA & 0.5 mg/l BAP. After 3 weeks the multiplied explants were transferred for shoot regeneration supplemented with 1.0 mg/l BAP+0.25 mg/l IAA+0.5 mg/l GA3 with combination of Mn & $CaCl_2$. To induce root formation, shoots (4–5 cm high) were transferred medium that contains half strength MS basal medium supplemented with 0.1 mg/l IAA and different combination of CaCl2 & Mn (2, 3, 5 mM). After 3 weeks, well-rooted plantlets were transferred to polyethene bags filled with autoclaved mixture of soil and soilrite (commercial hardening mixture) were maintained in the green house for 3-4 months under natural light with relative humidity of 90-100% and ambient temperature of 250C. Then these fully developed plantlets were transferred to the field. They need protection from wind damage, drought, birds and animals.

3. Result and Discussion:

The present studies have shown the development of inorganic compound tolerant plants of *Nicotiana tabacum* through in vitro culture method. To achieve this goal different concentration of Inorganic compounds ($CaCl_2$ & Mn) were used alone or in combination with one another. The experimental result indicated that the types of cytokine & auxin and their concentration significantly influenced the initial growth, multiplication, shooting and hardening.

Callus initiation began immediately after the transplant of *N. tabacum* leaf to MS medium supplemented with different concentration of $CaCl_2$ and Mn in presence of NAA (1 mg/l) and BAP (1 mg/l). The results showed that the percent of survivability with optimum with concentration of $CaCl_2.2H_2O$; 2mM, $CaCl_2.2H_2O$; 3mM, $CaCl_2$; 2mM + Mn 2mM and $MnSO_4$. H_2O; 2mM but concentration of Mn at 5mM, with $CaCl_2$: 2mM, with $CaCl_2$: 3mM shows toxicity. The Callus initiation was observed in cut surface of leaves after 15 to 18 days of culture initiation. Maximum amount of callusing (100%) was observed on the MS medium supplemented with combination of BAP 1.0 mg/l and NAA 1.0 mg/l with 2mM Inorganic compounds ($CaCl_2$ & Mn) (Fig-A). By contrast, the 5mM Mn treatment caused a significant decrease of callus induction with respect to the control. This observation is consistent with those reported by Petolino and Collins (1985) who found that callus derived from Mn-tolerant clones of tobacco were more tolerant to Mn than non-tolerant ones. Moreover, Kintzios et al. (2003) reported that callus cultures of mistletoe (Viscum album) derived from stem explants accumulated more Fe, Mn, Zn and Cu than calli derived from leaf explants. Celebration & Yao Genhuai (1992) suggests that Cytokinins is necessary for callus differentiation, and the ratio of the auxin to the cytokinin determining the type of culture established or regenerated. Shoot bud regeneration from callus was achieved from leaf tissues on MS media supplemented with 1.0 mg/l BAP+0.25mg/l IAA+0.5mg/l GA_3 and different concentration of $CaCl_2$ & Mn (Table-2). The results showed that the percentage survivability was about 82% in the MS medium containing 01.0 mg/L BAP +0.25 mg/l IAA+0.5 mg/l GA_3 and 3% sucrose. The percentage of survivability was decreased with increase of inorganic compound concentration from 2mM to 5mM. The use of growth regulators on production of shoot buds and subsequent plant regeneration of some grasses and cereals is well documented (Rueb et al., 1994; Rout et al., 1998). Inclusion of copper in the culture medium increases the rate of shoot bud regeneration. Low concentration of copper has been used as a microelement which plays an important role in the regeneration of plant tissue culture (Murashige and Skoog., 1962; Roustan et al., 1989; Sethi et al., 1990; Chraibi et al., 1991). The regeneration of shoot buds were maximum on

0.5 mg/l IAA+0.5 mg/l BAP medium as well as the medium containing 2mM $CaCl_2$ and 2mM Mn (Fig-B), However, initiation of multiple shoots in most of treatments was observed by rapidly sub culturing on similar medium (Fig C). The shoot proliferation and elongation were stronger in MS+0.25 mg/l IAA+1.0 mg/l BAP+0.5 mg/l GA_3 hormonal concentration with 2mM Mn and 2mM $CaCl_2$. Regenerated shoots (both tolerant and non tolerant) were transferred on to half strength MS basal medium supplemented with 0.01 mg/L IAA and different combination of $CaCl_2$ & Mn for induction of rooting (Fig D). The best rooting response, however, was observed on medium containing 0.01mg/l IBA with 2mM Mn and 2mM $CaCl_2$, where roots measuring 1.8± 0.4 cm (average) were formed (Table-3). After one week, about 92% of the tolerant plantlets were transferred to the green house and growing normally (Fig E & F). Non tolerant shoots became yellowish and root did not appear. Purnhauser and Gyulai (1993) and Wersuhn et al (1994) reported that copper and aluminium tolerant plants were developed in wheat, tobacco and potato respectively through in vitro system.

Table 1. Effect of growth medium 1 mg/ l NAA + 1 mg/l BAP + different concentrations of $CaCl_2$ and Mn) on callus formation from leaf disc explants of *N.tabacum* after 2 weeks of culture. The experiments were repeated three times. 15 replicates / treatment.

Mn & $CaCl_2$ Conc. (mM)		% of Survivabilit (Mean±S.D)	Av. No. of shoot buds per culture (Mean±S.D)	Av. length of the shoots per culture (Mean±S.D)
Mn	$CaCl_2$			
Control		85.6 ± 1.1	40.2 ± 0.8	1.4 ± 0.8
3	0	15.2 ± 1.1	2.3 ± 0.2	0.60 ± 0.01
2	2	82.07± 1.4	39.6 ± 0.7	1.2 ± 0.7
5	2	70.2± 0.9	28.4± 0.6	1.0 ± 0.4
2	3	63.3± 1.1	24.6 ± 0.8	0.56 ± 0.5
5	2	40.02± 0.1	18.03±0.02	0.32± 0.2

Table 2. Effect of growth medium 1.0 mg/L BAP +0.25 mg/L IAA+0.5 mg/L GA_3 + different concentrations of $CaCl_2$ and Mn on shoot bud regeneration and multiplication of *N.tabacum* after 4 weeks of culture. The experiments were repeated three times. 15 replicates/treatment.

Mn & $CaCl_2$ Conc.(mM)		% of Survivability (Mean± S.D)	Av. No. of shoot buds per culture (Mean±S.D)	Av. length of the shoots per culture (Mean± S.D)
Mn	$CaCl_2$			
Control		85.6±1.1	38.5 ± 0.3	1.0 ± 0.5
3	0	15.2 ± 1.1	35.09 ± 0.4	0.90 ± 0.3
2	2	82.07± 1.4	29.06±0.05	1.01 ± 0.9
5	2	70.2± 0.9	28.4± 0.6	1.0 ± 0.4
2	3	63.3± 1.1	21.2 ± 0.04	0.61 ± 0.3
5	2	40.02± 0.1	17.03±0.02	0.29± 0.02

Table 3. Effect of growth medium (1/2 MS + 0.1 mg/L IAA) on root induction of *N.tabacum* after 4 weeks of culture. The experiments were repeated three times.15 replicates/treatment.

Mn & $CaCl_2$ Conc. mM)		% of Survivability (Mean± S.D)	Av. No. of roots per culture (Mean±S.D)	Av. length of the shoots per culture (Mean± S.D)
Mn	$CaCl_2$			
Control		99.02±0.1	61.5 ± 0.3	2.1 ± 0.4
0	2	86.4 ±0.03	51.0 ± 0.4	1.4 ± 0.9
0	3	60.05 ±0	27± 0.03	0.8± 0.2
2	0	70.2 ±1.0	43.4± 0.8	1.01 ± 0.5
2	2	92.03±1.4	57.2 ± 0.05	1.8± 0.4
5	2	51.06±0.1	17.03±0.1	0.4± 0.06

4. Conclusion:

The present investigation was to develop the Mn & $CaCl_2$ tolerant plant through the modern tissue culture technique. The efficient regeneration system via. shoot bud proliferation from leave explants of *Nicotiana tabacum* showed high tolerance to Mn & $CaCl_2$. This protocol may be useful for genetic improvement of plant adaptation to the extreme environmental stress and also to the mechanism of Mn & $CaCl_2$ action on plant regeneration which help breeders to develop improved types.

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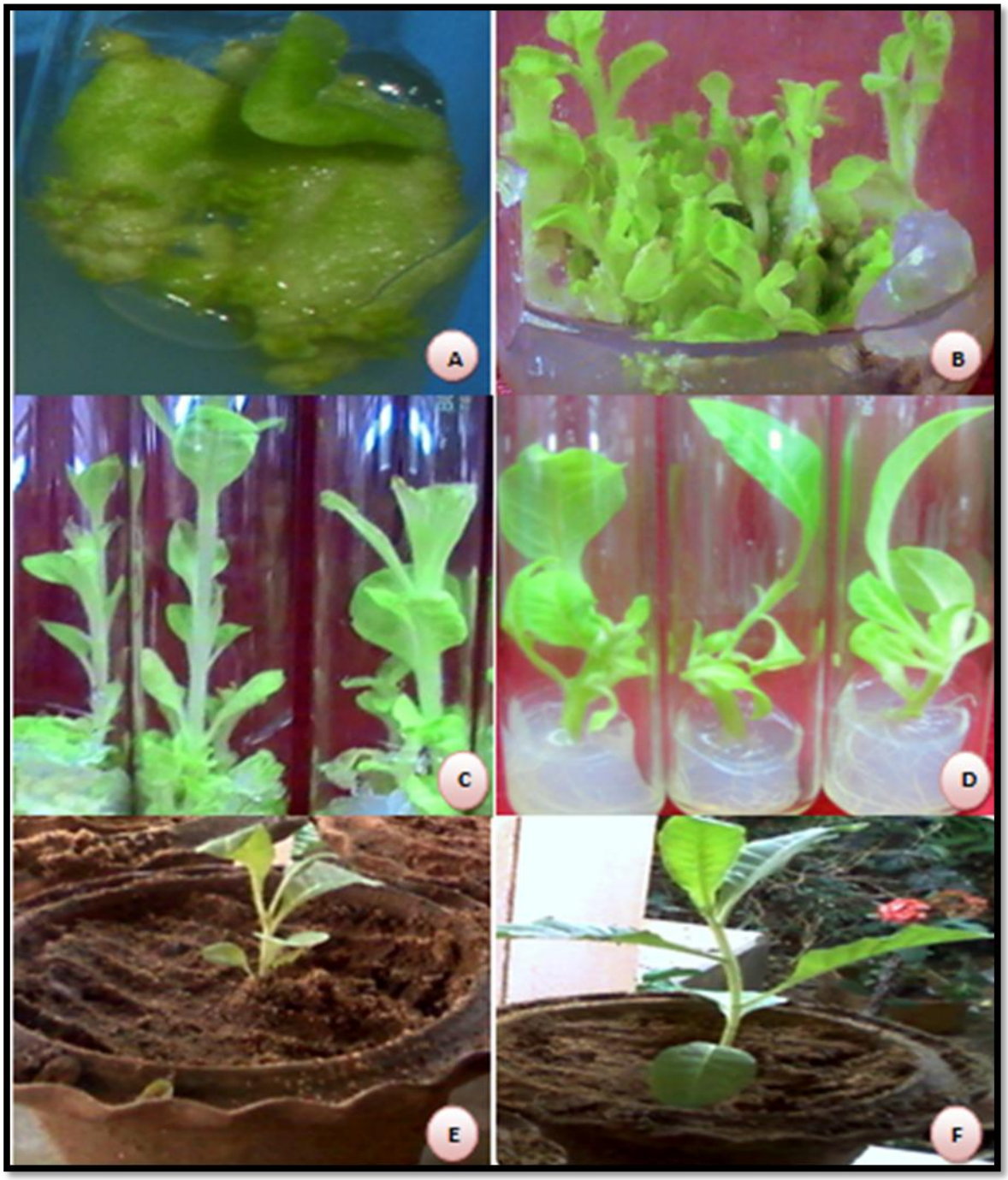

Figure: - Micropropagation of *N.tabacum* (A) Callus induction in MS supplemented with 1 mg/ l NAA + 1 mg/l BAP+ 2MM of Mn $CaCl_2$ **. (B)** Bud regeneration and multiplication from calli in MS supplemented with 1.0 mg/L BAP +0.25 mg/L IAA+0.5 mg/L GA3 + 2MM of CaCl2 and Mn . **(C)** Shoot Elongation in MS with 1.0 mg/L BAP +0.25 mg/L IAA+0.5 mg/L GA3 + 2MM of CaCl2 and Mn after 3 to 3 times of subculture . **(D)** Root formation on ½ MS with 1.0 mg/l IBA+2MM of CaCl2 and Mn . **(E &F)** Rooted plantlets transferred to pots for hardening under greenhouse conditions.

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