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RESEARCH **P**APER

An efficient protocol for *in vitro* regeneration in java citronella (*Cymbopogon winterianus*) through callus

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The reproducible indirect *in vitro* regeneration system through callus was developed in Java citronella (*Cymbopogon winterianus*) for genotype Bio-13. Tender leaves from the lower portion of the citronella culms were used as explants. The MS medium supplemented with different concentrations of 2, 4-D (1.0, 3.0, 5.0 and 7.0 mg/l) alone or in combination of BAP (0.2, 0.4 and 0.6 mg/l) were used for callus induction. For regeneration of plantlets MS media with different concentrations of BAP (0.1, 0.2, 0.3 and 0.5 mg/l) alone or in combination with NAA (0.1, 0.2 and 0.3 mg/l) were employed. The induction of organogenic callus was highest in medium containing 5 mg/l 2, 4-D with 99.66 per cent explants showing callus formation. At higher concentration of BAP (0.5 mg/l) shoots were initiated rapidly from the callus within 13 days. The root formation response was best in MS medium containing 3.0 mg/l NAA (83.33 % shoots formed roots within 25 to 30 days). The regenerated plantlets transferred to autoclaved garden soil, soilrite and sand in 1:1:1 proportion and irrigated with half strength MS solution showed 85 per cent survival rate after three weeks.

Key words : Callus, *In-vitro* regeneration, Aromatic grass, Citronella, Somaclonal variation

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INTRODUCTION

Development of superior lines in terms of oil and biomass production in crops is a major thrust area of aromatic crops. Java citronella (*Cymbopogon winterianus*) is a tall perennial, foliage rich aromatic grass belonging to family Poaceae and genus Cymbopogon. It is the source of the high quality java citronella essential oil. The demand for essential oil of citronella is increasing in the international market for its use in perfumery and cosmetic industry (Shiva *et al.*, 2002). Java citronella, which is known only in cultivation does not set viable seeds and is conventionally propagated by rhizomes (Sreenath and Jagadishchandra, 1989). The *in vitro* regeneration coupled with variation in percentage of essential oil will help in wide adaptation of citronella on farmer's field. The technique is also been used for genetic transformation studies undertaken for introduction of novel phenotypes, which is otherwise not possible to introduce through conventional breeding methods involving hybridization and subsequent selection or population improvement. As it is a vegetatively propagated crop, continuous clonal propagation leads to the accumulation of pests and pathogens leading to decline of vigour and quality. Improvement in the existing genotypes is major thrust area. Therefore, an optimization of protocol for *in vitro* culture through callusing with the aim to create the *in vitro* variability through somaclonal variation or *in vitro* mutation in the commercially grown genotype of java citronella to identify the stable clone/genotypes for high oil production is an important research area. To create variability through *in vitro* mutagenesis an efficient tissue culture based regeneration system is essential and therefore, the reproducible indirect *in vitro* regeneration system through callus in java citronella (*Cymbopogon winterianus*) for genotype Bio-13 is reported in this paper. This paper reports the results of studies designed to test different growth regulator formulations for the induction of callus and its organogenesis responses in tender leaf explants with aim to develop a long-term regenerable tissue culture system suitable for biotechnological improvement java citronella.

RESEARCH METHODOLOGY

This experiment was carried out at Plant Tissue Culture and Transformation Laboratory, Biotechnology Centre, PGI,

Dr. Punjabrao Deshmukh Krishi Vidyapeeth, Akola. The experimental material of present investigation comprised of popular variety of Java citronella. The clumps of citronella variety Bio 13 were sterilized with 0.1 per cent mercuric chloride and few drops of tween–20 for five minutes. The outer leaves were removed and innermost leaf whorls were inoculated in MS media fortified with various concentrations of 2, 4-D (1.0, 3.0, 5.0 and 7.0 mg/l) alone or in combination with different concentrations of BAP (0.2, 0.4 and 0.6 mg/l) in seventeen different combinations to obtain callus culture. The calli were inoculated on regeneration medium comprising of MS medium supplemented with BAP (0.1, 0.2, 0.3 and 0.5 mg/l) alone or in combination with NAA (0.1, 0.3 and 0.5 mg/l). The elongated multiple shoots regenerated from calli were transferred to full and half strength MS medium fortified with different concentration of IAA (0.5, 1, 2 and 3 mg/lit) and NAA (0.5, 1, 2 and 3 mg/l). The cultures were incubated at 25± 2^{0}C in dark with 60 to 70 per cent humidity for callus induction and under 12 hour daily illumination with cool white fluorescent tubes for plant regeneration.

Experimental designs and statistical analysis :

The experiment was conducted in Randomized Design with three replications. The data was analyzed using analysis of Variance for the Completely Randomized Design and treatment means were compared (Panse and Sukhatme, 1967). Each replication comprise of five culture bottles per treatment to study the effect of different media combination on *in vitro* regeneration of citronella. The plain MS medium served as control.

RESEARCH FINDINGS AND ANALYSIS

Observations were recorded on various parameters for callus induction, plant regeneration response, rooting induction and hardening response.

Callus induction :

16 different combinations of 2, 4-D and BAP were used along with MS plane as control. Observations were recorded on different parameters of callus induction as days to callus induction, callus induction response in percentage and callus proliferation after 45 days.

Number of days required for callus initiation after inoculation of explants :

Un-whorling of the leaves, along with swelling of the explant was observed after 4-5 days of culturing. The explant turned pale in colour. Callus initiation started after 15-18 days from the cut edges or injured sites and gradually callus growth occurs completely over the explant. Among the sixteen treatments and control tried for callus induction, it was observed

Table 1 : Response to callus induction on various culture media after inoculation of explants

Treatments	Media combination	Mean number of days required for callus initiation	Response to callus induction (%)	Mean callus proliferation after 45 days of initiation of callus (mg)
T_1	MS-control	0	0(0)	296.7
T_2	MS+2, 4-D 1 mg/l	18.6	86.66 (68.57)	803.2
T_3	MS+2, 4-D 1 mg/l + BAP 0.2 mg/l	18.0	80.00 (63.43)	774.2
T_4	MS+2, 4- D 1 mg/l + BAP 0.4 mg/l	18.1	73.33 (58.90)	755.7
T_5	MS+2, 4-D 1 mg/l + BAP 0.6 mg/l	17.6	70.00 (56.78)	723.8
T_6	MS+ 2, 4-D 3 mg/l	16.0	93.33 (75.03)	892.7
T_7	MS+2, 4-D 3 mg/l + BAP 0.2 mg/l	15.5	86.66 (68.57)	886.3
T_8	MS+2, 4-D 3mg/l +BAP 0.4mg/l	15.1	83.33 (65.90)	834.7
T_9	MS+2, 4-D 3 mg/l +BAP 0.6 mg/l	15.0	76.66(61.11)	808.8
T_{10}	MS+ 2, 4-D 5 mg/l	14.3	96.66 (79.46)	947.0
T_{11}	MS+2, 4-D 5 mg/l +BAP 0.2 mg/l	14.7	90.00 (71.56)	926.0
T_{12}	MS+2, 4-D 5 mg/l +BAP 0.4 mg/l	14.9	86.66 (68.57)	874.0
T_{13}	MS+2, 4-D 5 mg/l +BAP 0.6 mg/l	15.2	80.00 (63.43)	853.4
T_{14}	MS+2, 4-D 7 mg/l	18.8	60.00 (50.75)	498.4
T_{15}	MS+2, 4-D 7 mg/l+ BAP 0.2 mg/l	17.9	53.33 (46.90)	444.6
T_{16}	MS+2, 4-D 7 mg/l+ BAP 0.4mg/l	17.6	50.00(45.00)	392.6
T_{17}	MS+2, 4-D 7 mg/l+ BAP 0.6mg/l	17.5	43.33(41.60)	376.2
	F test	Sig.	Sig.	Sig.
	S.E. ±	0.30	0.72	1.75
	C.D. (P=0.05)	0.87	2.06	5.03

Figures in parenthesis are in arcsine values

that callus formation was observed in all treatment combination. Callus induction was not observed on MS taken as control without any growth hormone. Salehi *et al.* (2008) also reported that in absence of supplementary hormones there was no callus induction in control medium while working on bermuda grass. A treatment containing 5 mg/l 2, 4-D alone was found highly significant and required minimum number of days (14.3 days) to initiate callus. While the medium containing higher concentration of 2, 4-D (7 mg/l) required maximum days (18.8 days) to initiate callus. Cai and Butler (1990) has also reported inferior results for callus induction in sorghum on the media containing higher concentration of 2,4-D. Patnaik and Debata (1997) obtained calli of *Cymbopogon martini* within 12-15 days after the suspension culture was placed on medium supplemented with 13.6 µM 2, 4-D and 1.15 µM kinetin.

Callus induction response :

All the explants inoculated on control medium fails to turn in to callus. Thus, it was observed that presence of auxin (2, 4-D) is essential for callus induction in java citronella. Variable response for callus induction was recorded and the treatment MS + 2, 4-D 5 mg/l was found significantly superior with 96.66 per cent explants forming callus. Increased in the concentration of 2, 4-D, the response to the callus induction was also increased but it was at optimal level of 5 mg/l. However, with the increase in the concentration of BAP, it was observed that the callus induction decreased. As the concentration of 2,4-D was increased to 7 mg/l, the callus induction percentage was decreased (60%). This may be due to the interference of inhibitory compounds such as abcisic acid and many phenolics which often extracted along with auxin (Salisbury and Ross, 2009). Hence, higher concentration of 2, 4-D 7 mg/l alone or in combination with BAP (0.2 mg/l, 0.4 mg/l and 0.6 mg/l) showed poor response towards callus formation (60.00%, 53.33%, 50.00% and 43.33%, respectively). Similar results were obtained in different experiments conducted by various researchers. Sikder *et al.* (2006) noticed that MS media supplemented with 1.5, 2.0, 2.5, and 3.0 mg/l 2, 4-D produce 100 per cent callus and MS medium supplemented with 0.5 mg/l 2,4-D produced lowest percentage (40%) of callus during the *in vitro* regeneration of aromatic rice. Jain *et al.* (2005) found maximum callus induction in *Cynodondactylon* with 88 per cent of explants responding on medium containing 4 mg/l 2,4-D followed by 66 per cent on 2,4-D 3 mg/l, respectively. The callus formed on lower concentration of 2, 4-D (1 mg/l) was non compact, mucilaginous and watery, whereas compact, nodular creamish white callus was formed on medium supplemented with 3 mg/l and 5 mg/l 2, 4-D.

Callus proliferation after 45 days of initiation of callus :

It was observed that though high concentration of 2, 4-

Table 2 : Response of callus to shoot induction

Tr.	Media combination	No. of calli showed regeneration	Response to shoot induction (%)	Mean no. of days required for shoot initiation	Mean no. of days required for multiple shoot induction	Mean no. of multiple shoot obtained after 30 days
T_1	MS-control	9	30.00 (33.21)	19.2	22.5	06.0
T_2	MS+BAP 0.1mg/l	24	80.00 (63.43)	15.6	15.4	51.3
T_3	MS+BAP 0.2 mg/l	26	90.00 (71.56)	15.2	15.2	52.3
T_4	MS+BAP 0.3 mg/l	28	93.33 (75.03)	14.6	15.0	53.9
T_5	MS+BAP 0.5 mg/l	29	96.66 (79.04)	13.0	14.0	56.8
T_6	MS+BAP 0.1+ NAA 0.1mg/l	25	83.33 (65.90)	16.4	16.8	22.4
T_7	MS+BAP 0.1+ NAA 0.3 mg/l	22	73.33 (58.90)	17.6	21.0	22.2
T_8	MS+BAP 0.1+ NAA 0.5 mg/l	21	70.00 (56.78)	18.4	21.1	19.2
T_9	MS+BAP 0.2+ NAA 0.1mg/l	21	70.00 (56.78)	17.1	17.4	25.3
T_{10}	MS+BAP 0.2+ NAA 0.3 mg/l	19	63.33 (52.03)	18.3	17.4	19.5
T_{11}	MS+BAP 0.2+ NAA 0.5 mg/l	23	76.66 (61.11)	19.2	21.1	20.8
T_{12}	MS+BAP 0.3+ NAA 0.1mg/l	26	86.33 (68.80)	16.8	16.5	26.4
T_{13}	MS+BAP 0.3+ NAA 0.3 mg/l	25	83.33 (65.90)	17.0	19.3	19.3
T_{14}	MS+BAP 0.3+ NAA 0.5 mg/l	21	70.00 (56.78)	19.4	21.7	24.0
T_{15}	MS+BAP 0.5+ NAA 0.1mg/l	28	93.33 (75.03)	17.2	16.1	28.5
T_{16}	MS+BAP 0.5+ NAA 0.3 mg/l	24	80.00 (64.43)	18.2	19.3	30.1
T_{17}	MS+BAP 0.5+ NAA 0.5 mg/l	25	83.33 (65.90)	17.8	21.7	19.2
	F test		Sig.	Sig.	Sig.	Sig.
	S.E. $\pm$		0.74	0.27	0.47	0.88
	C.D. (P=0.05)		2.14	0.77	1.35	2.56

D is required for callus induction but multiplication of callus is carried out at reduced or without 2, 4-D. Leaf explants from the lower portion of the culms formed morphogeniccalli more readily than the leaves from middle portion of the culm, with most prolific callus formation in the dark. A considerable mass of callus was accumulated within 40-45 days. Established callus cultures were then multiplied for two cycles by serial sub-culturing in multiplication media. Sub-culturing was done regularly at an interval of 21 days. The initial fresh weight of three replicates each for seventeen treatments of callus was taken.

The growth of callus was monitored by fresh weight measurements, after 45 days of initiation of callus. It was observed that all the medium combination showed a consistent increase up to 21 days. Also the proliferation of callus was significantly superior in 2, 4-D 5 mg/l (947.0 mg). The weight of callus increases with increase in the concentration of 2, 4-D at optimal level of 5 mg/l. The lowest weight (296.7 mg) was recorded in MS plain (control). Cai and Butler (1990) reported inferior results to the media containing higher concentration of 2, 4-D. Tariq *et al.* (2008) obtained 0.22, 0.27, 0.25 gm mean weights of three rice varieties on media with 2.5 mg/l 2, 4-D and highest mean weight 0.26 gm was observed on medium supplemented with 2, 4-D 3 mg/l. Ramesh *et al.* (2009) reported maximum response in terms of percentage of callus induction (76.6%) and highest mean fresh weight (206.6± 0.39) on medium supplemented with 2, 4-D 11.3 µM in Indica rice.

Plant regeneration medium :

After 45 days of callus initiation the fresh weight of calli were taken. These calli were cut into small pieces and cultured on MS regeneration medium supplemented with different combinations and concentrations of growth regulators, including BAP (0.1, 0.2, 0.3, 0.5 mg/l) alone or in combination with NAA (0.1, 0.3 and 0.5 mg/l). The calli when transferred to the media containing cytokinin alone, showed best response in terms of shoot induction. The medium containing MS plus BAP 0.5 mg/l was highly superior and 96.66 per cent callus in this medium showed regeneration. Sikder *et al.* (2006) reported highest regeneration frequency of callus (100 %) in chiniguri variety of rice in medium supplemented with 0.05 mg/l NAA and 5.0 mg/l BA. Nayak (1996) reported shoot regeneration from the callus of *Cymbopogon* species by transferring to the MS medium supplemented with NAA 0.5 mg/l, kinetin 1 mg/l and BA 1 mg/l.

It was observed that in control treatments, shoot proliferation rate was significantly lower than the other mediums containing different concentrations of cytokinin indicating significant role of cytokinin in shoot multiplication. Only six shoot were regenerated from callus in MS medium devoid of any hormone. The maximum number of shoots (56.8 shoots) obtained on medium containing higher level of BAP 0.5 mg/l which showed highly significant results over other

Table 3 : Root induction response and hardening

Treatments	Media combination	Root induction response (%)
T_1	MS (control)	0 (0)
T_2	MS + IAA 0.5 mg/l	40.00(39.23)
T_3	MS + IAA 1.0 mg/l	46.60(43.05)
T_4	MS + IAA 2.0 mg/l	50.00(45.00)
T_5	MS + IAA 3.0 mg/l	33.33(35.24)
T_6	½ MS + IAA 0.5 mg/l	66.66(54.73)
T_7	½ MS + IAA 1.0 mg/l	73.33(58.90)
T_8	½ MS + IAA 2.0 mg/l	80.00(63.43)
T_9	½ MS + IAA 3.0 mg/l	33.33(35.26)
T_{10}	MS + NAA 0.5 mg/l	40.00(39.23)
T_{11}	MS + NAA 1.0 mg/l	46.66(43.08)
T_{12}	MS + NAA 2.0 mg/l	56.66(48.80)
T_{13}	MS + NAA 3.0 mg/l	83.33(65.11)
T_{14}	½ MS + NAA 0.5 mg/l	76.66(61.11)
T_{15}	½ MS + NAA 1.0 mg/l	66.66(54.73)
T_{16}	½ MS + NAA 2.0 mg/l	60.00(50.76)
T_{17}	½ MS + NAA 3.0 mg/l	50.00(45.00)
	F test	Sig.
	S.E. ±	0.64
	C.D. (P=0.05)	1.86

treatments. The number of shoots decreased in medium supplemented with higher concentration of NAA. Denchev and Conger (1995) obtained 45 shoots per callus in medium supplemented with 22.3 µM (5mg/l) picloram with 5.0µM (1 mg/l) BA, during *in vitro* regeneration of switchgrass. Nayak *et al.* (1996) obtained approximately 30 to 35 plantlets when 100 mg callus of *C. flexuosus* was cultured on medium containing 3 mg/l BA, 1 mg/l GA_3 and 0.1 mg/l BA. Hu *et al.* (2006) found 0.9 µM 2,4-D and 4.4 to 8.9 µM BA most suitable for multiple shoot induction and obtained 15 to 20 shoots from each clump kentucky bluegrass.

Root induction response :

Elongated multiple shoots regenerated from calliwere excised from each culture passage and transferred to full and half strength MS medium supplemented with different concentrations of IAA (0.1, 1, 2, 3 mg/l) and NAA (0.1, 1, 2, 3 mg/l).

From the Table 3, the best root formation response was obtained in full strength MS media containing 3.0 mg/l NAA. At this concentration 83.33 per cent shoots formed roots within 25 to 30 days of inoculation. Metzinger *et al.* (1987) observed root development by transferring the young shoots of bluestem grass to half-MS medium containing 1 mg/l NAA. The rooting of the regenerated shoots of sorghum on MS medium supplemented with 10.7 µM NAA were reported by Nirwan and Kothari (2003). Hu *et al.* (2006) observed rooting to the shoots on medium without or only supplemented with 0.5 µM NAA in Kentucky bluegrass. Be *et al.* (2008) obtained an average nineteen roots per clump when the shoots of *Vetiveria zizanioides* were transferred to medium containing 1 mg/l NAA.

Hardening of the regenerated plants :

The rooted plants transferred to soilrite and irrigated with half strength MS solution showed better survival rate (85%) after 3 weeks. However, the plants which were irrigated with tap water showed only 30 per cent survival rate under the green house conditions.

The plantlets with well developed roots were removed from the culture medium and washed under running tap water to remove the agar sticking to them. These plantlets were treated with bavistin solution (2%) and again washed with water. Finally the plantlets were transferred to plastic pots containing autoclaved garden soil, soilrite and sand in 1:1:1 proportion and half of them were irrigated with water and other half irrigated with half MS solution. The potted plantlets were covered with porous polythene sheets for maintaining high humidity and were maintain in the culture room. After 15 days the plantlets were transferred under shade in a net house for further growth and development.

It was observed that the plants which were irrigated with half strength MS solution showed better survival rate (85%) after 3 weeks. However, the plants which were irrigated with tap water showed only 30 per cent survival rate under the green house conditions. Similar results were also reported by Metzinger *et al.* (1987) the survival rate exceeding at 90 per cent when plantlets of bluestem grasses were transferred to the potting soil. Sreenath and Jagadishchandra (1989) reported 100 per cent survival of *C. winterianus* for 10-15 days in greenhouse condition.

Abbreviations :

BA–6–benzylaminopurine;
2, 4-D – 2, 4-dichlorophenoxy acetic acid;
IAA – indole acetic acid;
Kin – kinetin;
MS – Murashige and skoog medium (Murashige and Skoog, 1962).

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