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Somaclonal variation among somatic embryo derived plants — Evaluation of agronomically important somaclones and detection of genetic changes by RAPD in *Cymbopogon winterianus*

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ABSTRACT

A total of one hundred five plants of *Cymbopogon winterianus* derived from indirect somatic embryogenesis were established in the field. Extensive somaclonal variation was noticed within these plants for all eight agronomic characters such as plant height, diameter of bush, number of tiller/clump, number of leaves/clump, leaf length, leaf breadth, weight of 100 leaves and oil content in relation to the donor parent. Significant variations were also recorded for two major constituents of essential oil such as citronellal and geraniol in some selected somaclones. Ten somaclones that were retained improved oil content and quality in initial screening and, in a replicated trial, were further assessed for their stability in the field for four clonal propagations. Out of the ten superior somaclones only five superior somaclones (SC1, SC2, SC3, SC6 and SC10) which showed relative stability both in oil content and quality were subjected to random amplified polymorphic DNA (RAPD) analysis. Out of the eighteen primers used, ten primers revealed polymorphism showing distinctly different banding patterns in the five improved somaclones, which were equally prominent in their differences from the control. Out of the 44 polymorphic bands, 30 were parental bands which were missing from some somaclonal progenies and 14 were novel non-parental bands which were detected only in some somaclones that confirmed the presence of genetic changes due to somaclonal variation.

1. Introduction

Cymbopogon winterianus Jowitt, commonly known as citronella Java, is a vegetative propagated perennial grass due to extremely poor seed setting and low germination percentage. Predominant vegetative mode of cultivation precludes the possibility of applying a traditional breeding system based on sexual mating and recombination for the genetic improvement of this grass. The species shows considerable heterogeneity in the seed grown natural population due to outbreeding (Sreenath and Jagadishchandra, 1991), which apparently has an important bearing on the yield and quality of the essential oil. Moreover, from the literature it appears that this particular species shows a wide range of chromosomal variations in essential composition, which was encountered in clonal progenies (Lavania, 1988). This has resulted in a serious problem in the aromatic industry causing the deterioration of existing varieties with respect to herbage yield, oil yield, oil content, oil quality and resistance to diseases (Hussain, 1982). An alternative method for developing superior strains of this grass is by selecting somaclonal variants from tissue culture derived plants. It is a fact that the in vitro generated variations, known as somaclonal variation (Larkin and Scowcroft, 1981), affect the genetic fidelity of the regenerated plants and at the same time, also serve as a rich source of genetic diversity for the introduction of novel traits into a wide range of crops (Bajaj, 1990; Peschke and Phillips, 1992; Jain, 2001). The occurrence of somaclonal variation only through callus culture has been demonstrated in three species of *Cymbopogon*—*C. winterianus*, *Cymbopogon martinii*, and a hybrid *Cymbopogon*-Jamrosa (Marhur et al., 1988; Patnaik et al., 1999; Nayak et al., 2003).To the best of our knowledge, no study on somaclonal variation in plants of citronella Java regenerated via somatic embryogenesis has been made before. We have earlier reported the protocol of high frequency plantlet regeneration through somatic embryogenesis from rhizome explants for mass scale propagation of this plant (Dey et al., 2010a). However, as evidenced from the literature, screening and evaluation of somaclonal variants with improved oil yield and quality derived from callus culture in citronella Java have been reported for the first time by Marhur et al. (1988), but molecular markers were not used to establish the genetic basis of the somaclonal variation. Various molecular markers are widely used to detect and characterize somaclonal variation at the DNA level (Ford-Lloyd et al., 1992). Of the available techniques, Random Amplified Polymorphic DNA (RAPD) is mostly used (Saker et al., 2000; Jin et al., 2008; Peyvandi et al., 2010; Aruingtyas et al., 2012) as it offers the advantage of being simple to use, less expensive and less time consuming than Restriction Fragment Length Polymorphism (RFLP) and

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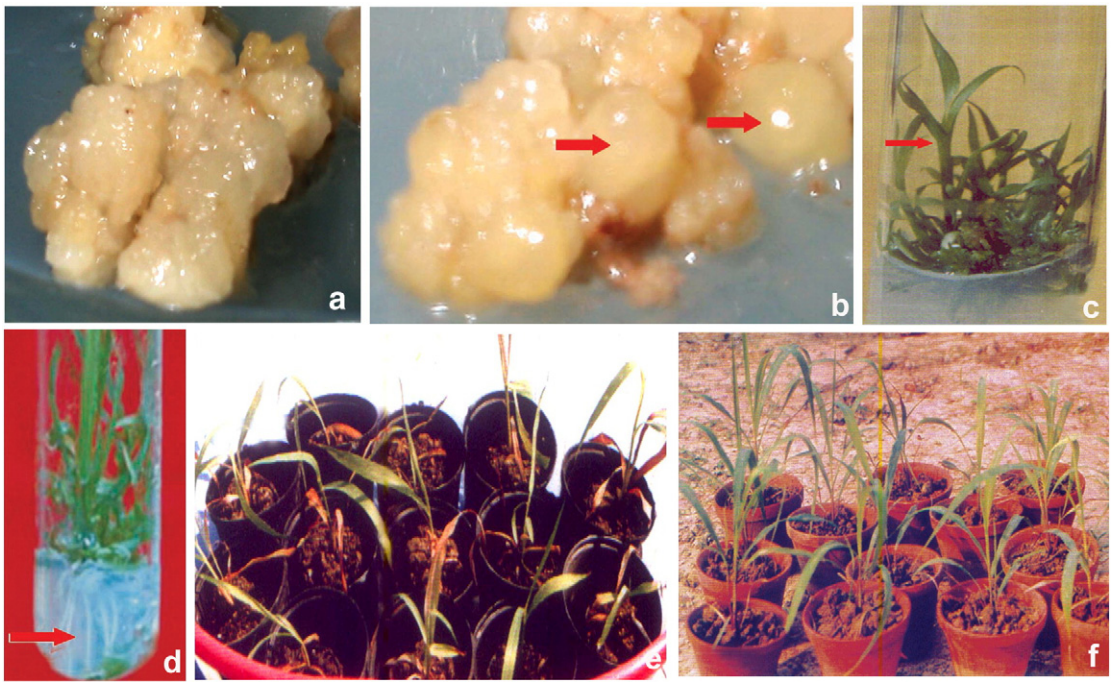

Fig. 1. In vitro propagation of *Cymbopogon winterianus* through indirect somatic embryogenesis using rhizome segments. (a) Embryogenic callus formation from rhizome explants on MS + 1.5 mg/l 2, 4-D + 0.5 mg/l Kn; (b) appearance of distinct globular embryos from embryogenic callus clumps on MS + 3 mg/l 2, 4-D + 0.5 mg/l Kn + 10% coconut water (*v*/*v*); (c) shoot proliferation on MS + 2 mg/l BA + 0.5 Kn/l + 0.2 mg/l CaP + 0.2 mg/l biotin; (d) rooted plantlets on MS + 1 mg/l IBA; (e) hardened plantlets in the green house; and (f) field transferred plants.

Amplified Fragment Length Polymorphism (AFLP). Therefore, in order to evaluate the genetic changes of somaclonal variants regenerated through somatic embryogenesis, RAPD was used in the present study. This communication sets out to enumerate the results of our efforts pertaining to the development of somaclonal variants, the quantitative and qualitative analysis of selected somaclones in the field, screening and selection of agronomically useful somaclones with high oil yield and quality and detection of gross genetic changes at DNA level through RAPD analysis.

2. Materials and methods

2.1. Plant material

Intact rooted slips of *C. winterianus* were collected by the courtesy of Regional Research Laboratory, Bhubaneswar, Orissa, India and used as the source of explants.

2.2. Somatic embryogenesis and plantlet regeneration

Plantlets were regenerated through indirect somatic embryogenesis from rhizome derived callus of *C. winterianus* (Dey et al., 2010a). The somatic embryogenesis consisted of induction of callus from rhizome explants on MS (Murashige and Skoog, 1962) basal medium supplemented with varying concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D), subsequent subculturing on MS nutrient medium containing 2, 4-D and Kinetin (Kn), transfer of callus clumps to embryogenic medium supplemented with 2, 4-D, Kn and coconut water, and subculturing at monthly intervals into the same freshly prepared medium. For plantlet regeneration, the embryos were transferred to MS medium containing N^6-benzyladenine (BA), Kn, calcium pantothenate (CaP) and biotin. Roots were formed when regenerated shoots were transferred to MS basal medium (1/2 strength) supplemented with different combination and concentrations of rooting hormones like α-naphthalene acetic acid (NAA) and indole-3butyric acid (IBA).

2.3. Establishment of plantlets in soil

The callus derived plantlets were transferred to small earthen pots and hardened in glass house under high humidity (90–95% RH) for 10–15 days at 25–32 °C. Initially they were irrigated with Hoagland and Arnon (1950) nutrient solution for 1 week. The established plants were then shifted to soil in big earthen pots and kept under observations in a green-house for approximately 4–6 weeks. A total of 118 plants were then transferred to the field and planted in rows with 90 cm

Table 1
Comparison of agronomic characteristics between propagated plants (control) and in vitro raised original somaclones.

Characters	Range of variations		Mean ± SD		CV%	
	Somaclones	Control	Somaclones	Control	Somaclones	Control
Plant height (cm)	80–219	110–195	150.23 ± 36.21NS	144.3 ± 22.87	24.10	15.84
Diameter of bush (cm)	21–127	33–85	79.26 ± 29.63*	56.8 ± 17.69	37.38	31.14
Number of tiller/clump	20–105	35–72	67.11 ± 23.01*	52.5 ± 10.72	34.29	20.42
Number of leaves/clumps	210–551	225–449	378.9 ± 101.47*	307.6 ± 63.52	26.78	20.65
Leaf length (cm)	60–166	85–119	115.28 ± 22.62NS	102.7 ± 12.5	19.62	12.17
Leaf breadth (cm)	1.2–2.8	1.8–2.4	2.118 ± 0.364NS	2.143 ± 0.179	17.19	8.35
Wt. of 100 leaves (g)	278–385	305–341	332.69 ± 26.39NS	324.3 ± 11.78	7.93	3.63
Oil content (%)	0.85–3.2	0.9–1.45	1.967 ± 0.69*	1.112 ± 0.1766**	35.08	15.88

*Significantly different from control ($P < 0.05$). **$P < 0.01$); NS (not significant) (Student's t-test).

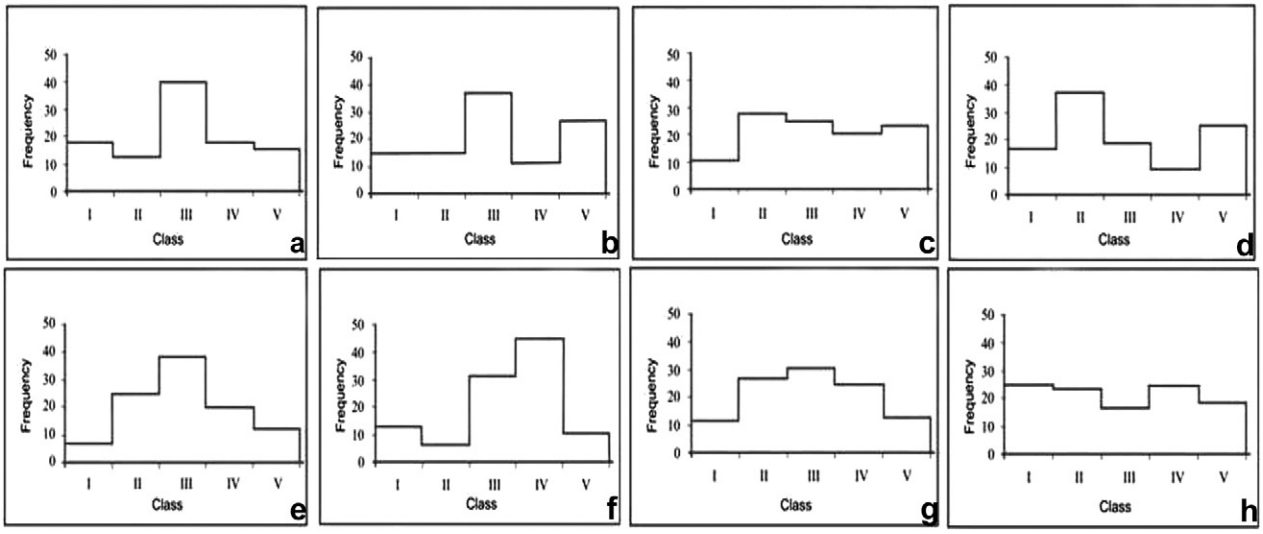

Fig. 2. Frequency distributions of eight agronomic traits assessed in the initial screening of 105 somaclones. (a) Frequency distribution of plant height (cm); (b) frequency distribution of diameter bush (cm); (c) frequency distribution of number of tiller/clump; (d) frequency distribution of number of leaves/clump; (e) frequency distribution of leaf length; (f) frequency distribution of leaf breadth; (g) frequency distribution of weight of 100 leaves; and (h) frequency distribution of oil content (% fresh weight basis).

spacing between the rows and 60 cm spacing between plants. A row of donor plants of the same age was planted in the field to serve as control.

2.4. Screening of somaclones

Data of various agronomic traits namely plant height, diameter of bush, number of tiller/clump, number of leaves/clump, leaf length, leaf breadth, weight of 100 leaves and oil content (%) were recorded individually 120 days of growth in the field. The mean, standard deviation and range for different agronomic traits were analyzed. Data were classified into five continuous class intervals and then frequency distribution was tabulated. Oil contents were obtained by steam distillation of fresh herbs of field grown plants (somaclones) using Clevenger's (1928) apparatus following the method of Guenther (1972). Based on initial

Table 2
Agronomic characteristics of 10 selected somaclones of *C. winterianus* in initial screening.

Characters	Somaclone number										Control
	SC1	SC2	SC3	SC4	SC5	SC6	SC7	SC8	SC9	SC10	
Plant height (cm)	196	175	201	162	188	205	190	167	179	185	148
Diameter of bush (cm)	114	85	118	98	108	120	88	83	95	113	74
No. of tiller/clump	96	77	89	79	85	99	86	76	74	90	54
No. of leaves/clump	541	548	535	515	499	532	521	506	490	539	322
Leaf length (cm)	150	132	152	136	145	155	142	127	130	140	103
Leaf breadth (cm)	2.21	2.65	2.37	2.46	2.55	2.41	2.70	2.29	2.49	2.38	2.10
Weight of 100 leaves	371	345	378	335	340	358	360	329	338	351	315
Oil content (% fresh wt. basis)	3.01	2.85	3.12	2.59	2.74	2.91	2.86	2.66	2.35	2.98	1.21

Table 3
Means ± SD and their statistical significance for eight agronomic traits of 10 somaclones in relation to the donor parent in replication trial (no. of replicates—3).

Somaclone number	Plant height (cm)	Diameter of bush (cm)	No. of tiller/clump	No. of leaves/clump	Leaf length (cm)	Leaf breadth (cm)	Wt. of 100 leaves (g)	Oil content (%)
SC1	$192.33^{c} \pm 10.79$	$115.67^{a} \pm 7.51$	$101.33^{a} \pm 10.50$	$560^{ab} \pm 13.23$	$163.33^{a} \pm 10.41$	$2.23^{e} \pm 0.08$	$384.33^{ab} \pm 11.93$	$3.06^{ab} \pm 0.10$
SC2	$168.33^{de} \pm 7.64$	$90.33^{b} \pm 4.04$	$77.33^{bcd} \pm 16.07$	$567.67^{a} \pm 7.64$	$132.33^{d} \pm 11.02$	$2.73^{a} \pm 0.08$	$343.67^{d} \pm 8.08$	$2.84^{bcd} \pm 0.13$
SC3	$210.33^{b} \pm 1.53$	$120.67^{a} \pm 12.10$	$92.33^{abc} \pm 14.84$	$545^{bcd} \pm 6$	$156.67^{abc} \pm 9.02$	$2.36^{cd} \pm 0.10$	$386^{a} \pm 9.54$	$3.22^{a} \pm 0.13$
SC4	$162.67^{e} \pm 2.52$	$93.33^{b} \pm 5.69$	$74.67^{d} \pm 9.50$	$512.33^{ef} \pm 12.06$	$138.33^{d} \pm 8.74$	$2.47^{bc} \pm 0.13$	$334^{d} \pm 4.04$	$2.56^{e} \pm 0.13$
SC5	$186^{c} \pm 5.29$	$94.67^{b} \pm 3.06$	$84^{abcd} \pm 13.11$	$530.33^{cde} \pm 10.50$	$143^{cd} \pm 11.36$	$2.55^{b} \pm 0.10$	$333.33^{d} \pm 3.79$	$2.67^{e} \pm 0.13$
SC6	$219.6^{a} \pm 1.53$	$124.33^{a} \pm 7.77$	$95.67^{a} \pm 14.50$	$552.33^{fg} \pm 11.24$	$160.67^{ab} \pm 7.37$	$2.48^{bc} \pm 0.12$	$367.33^{bc} \pm 15.18$	$2.99^{bc} \pm 0.14$
SC7	$193^{c} \pm 2.65$	$85.67^{bc} \pm 6.43$	$85.67^{abcd} \pm 10.02$	$502^{abc} \pm 13.75$	$138.67^{d} \pm 13.05$	$2.75^{a} \pm 0.09$	$365.33^{c} \pm 10.50$	$2.77^{cde} \pm 0.16$
SC8	$159^{ef} \pm 3.61$	$77.67^{cd} \pm 7.64$	$77^{bcd} \pm 6.24$	$526^{de} \pm 10.58$	$131.67^{d} \pm 12.34$	$2.33^{de} \pm 0.06$	$339.67^{d} \pm 7.664$	$2.66^{de} \pm 0.15$
SC9	$175^{d} \pm 4.58$	$92.33^{b} \pm 7.02$	$75^{cd} \pm 5.57$	$485^{g} \pm 15$	$132.33^{d} \pm 8.08$	$2.47^{bc} \pm 0.08$	$337.67^{d} \pm 7.51$	$2.33^{f} \pm 0.08$
SC10	$194^{c} \pm 6.56$	$115.33^{a} \pm 9.45$	$93.67^{ab} \pm 19.18$	$547.33^{abc} \pm 5.51$	$146^{bcd} \pm 10.54$	$2.43^{bcd} \pm 0.08$	$371.67^{abc} \pm 10.41$	$3.10^{ab} \pm 0.08$
Donor	$151.67^{f} \pm 3.51$	$73.33^{d} \pm 3.06$	$55.33^{e} \pm 4.51$	$310^{h} \pm 13.23$	$101.67^{e} \pm 6.11$	$2.08^{f} \pm 0.08$	$310^{e} \pm 10$	$1.15^{g} \pm 0.06$
Total	182.91 ± 21.36	98.48 ± 18.10	82.91 ± 15.79	512 ± 70.12	140.42 ± 18.60	2.45 ± 0.21	352.12 ± 24.71	2.66 ± 0.55
MS of Clones df = 10	1397.67**	934.49**	501.74**	15458.69**	886.01**	0.12**	1755.75**	0.94**
Error MS df = 20	29.63	41.21	82.70	123.95	69.92	0.00	95.58	0.01
CD (0.05)	9.27	10.93	15.49	18.96	14.24	0.12	16.65	0.21
CD (0.01)	12.65	14.91	21.13	25.86	19.43	0.16	22.71	0.28

Means ± SE, means followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan, 1955).
** Significantly different from donor ($p < 0.01$).

screening only 40 plants which have performed equally or better than the parent plants in terms of the total oil yield were assessed for oil quality.

2.5. GC analysis of oil

GLC analysis of essential oil was performed on a GC-Agilent 6890$^{series\ plus}$ using capillary column DB-624 of 30 m × 0.53 m × 0.30 μm. Injector and detector temperature was maintained at 310 °C during analysis. Helium was used as carrier gas at an inlet pressure of 15 psi. The essential oil extracted from regenerated plants was compared with control as concerns the qualitative changes in oil constituents such as geraniol and citronellal.

2.6. Replicated trial of the selected somaclones

Superior somaclones showing an increase in oil content compared to the control also containing good quality of oil further evaluated in replicated trial. Selected somaclones were planted in Randomized Block Design (RBD) and data were collected on row basis taking the mean of three plants from a row. The somaclones which maintained an increased oil content and quality in both initial screening and replicated trial were selected and studied for clonal propagation during successive years. Stable somaclonal variants were subjected to RAPD analysis for detection of any genetic changes.

2.7. DNA extraction

DNA was extracted from young leaves and rhizome of regenerated plants and the donor plants by cetyltrimethyl ammonium bromide (CTAB) based procedure with minor modifications (Dey et al., 2010b). DNA was isolated at least three times and the quantity and quality of the extracted DNA samples were estimated by comparing band intensities on 0.8% agarose gel.

2.8. PCR based amplification

The DNA samples were subjected to RAPD assay using 10 arbitrary decamers (out of 18 primers tested) procured from Genie, Bangalore, India, with GC content 50–70%. The amplification reaction mixture (25 μl) containing 200 μM each of dNTPs, 1.5 mM of $MgCl_2$ and 10 pmol of decamer primers, 0.2 unit Taq polymerase (Genie), 2.5 μl of *Taq* buffer and 30 ng of genomic DNA. The amplification was carried out in a thermal cycler (Perkin Elmer, Model-2400) with an initial denaturation at 94 °C for 5 min. PCR was run for 40 cycles containing denaturation of template DNA at 94 °C for 1 min, primer annealing at 35 °C for 2 min and primer extension at 72 °C for 2 min. At the end there was a final amplification period at 72 °C for 5 min and subsequently the temperature brought down to 4 °C.

2.9. Similarity index

Reproducible bands were scored manually as '1' or '0' for the presence or absence of the bands. Data were analyzed and similarity matrices were constructed from the binary data with Jaccard's coefficients (Jaccard, 1908) and compared by using Mantel matrix-correspondence test using NTSYS pc software 2.1 (Rohlf, 2000). The efficiency of the primers was assessed on the basis of the three criteria, viz., i) percentage of polymorphic bands, ii) polymorphic information content (PIC) values, ranging between 0.0 and 0.5, and iii) marker index (MI). Polymorphic information content (PIC) and marker index (MI) for the RAPD primers were calculated according to the simplified formulae of Powell et al. (1996) as follows: $PIC = \sum 2F\ (1 - F)$, where, F = proportion of bands per assay units, n = number of loci detected, MI = polymorphic information content (PIC) × proportion of polymorphic bands × average number of loci per assay unit.

3. Result and discussion

3.1. Somatic embryogenesis and establishment of plantlets in the field

In vitro regeneration protocol through indirect somatic embryogenesis from rhizome explants of *C. winterianus* was standardized in our earlier report (Dey et al., 2010a) and was used for mass scale production of plants through somatic embryogenesis in vitro (Fig. 1). Plantlets were regenerated from embryogenic callus clumps with globular embryos. The regenerated plantlets of *C. winterianus* were rooted and the frequency of rooting was the highest (80%) on MS (1/2 strength) medium supplemented with 1 mg/l of IBA (Fig. 1d). After rooting, in vitro regenerated plantlets were eventually transplanted to earthen pots placed in the green house and then to the field. Following transfer plantlets require a minimum of 10–15 days hardening period under high humidity (90–95% RH) at 14–16 h day light and 25–32 °C temperature and the best season (under West Bengal climate) for their higher survival and a high frequency establishment in pots was between middle of July to late September (Fig. 1e). The frequency of establishment during the above mentioned season was 79.73% and this can be justified by the high percentage (83%) of establishment from callus derived plantlets of citronella Java (Marhur et al., 1988).Embryogenic callus derived plants were then shifted to the field and a high establishment percent (88.95%) was achieved (Fig. 1f).

3.2. Screening and selection of somaclones

Somaclones derived through somatic embryogenesis were compared with field grown clones on the basis of various agronomic characters. Overall screening of the data recorded on individual 105 somaclones has indicated a wide range of variations for all eight agronomic characters in relation to the donor parent. From Table 1 it is clear that range of variation, standard deviation, and coefficient of variations for all eight characters in the somaclones were higher in contrast to the

Table 4
Performance of 10 superior somaclones in replicated trial (RDB) data expressed over the donor parent which is taken as 100.

Characters	Somaclone number										Control
	SC1	SC2	SC3	SC4	SC5	SC6	SC7	SC8	SC9	SC10	
Plant height (cm)	126.80	110.98	138.68	107.25	122.67	144.83	127.24	104.83	115.38	127.91	100
Diameter of bush (cm)	157.73	123.19	164.56	127.27	129.10	169.55	116.83	105.92	125.91	157.28	100
No. of tiller/clump	183.14	139.76	166.88	134.96	151.82	172.91	156.62	139.17	135.56	169.29	100
No. of leaves/clump	180.65	183.12	175.81	165.27	171.08	178.17	161.94	169.68	156.45	176.56	100
Leaf length (cm)	160.65	130.16	154.10	136.07	140.65	158.03	136.39	129.51	130.16	143.60	100
Leaf breadth (cm)	107.21	131.25	113.46	118.75	122.60	119.23	132.21	112.02	118.75	116.83	100
Weight of 100 leaves (g)	123.98	110.87	124.52	107.85	107.53	118.49	117.85	109.57	108.92	119.87	100
Oil content (%)	266.09	246.96	280	222.61	232.17	260	240.87	231.30	202.61	261.74	100

Table 5
Percentage composition of two major constituents of oil (citronellal and geraniol) of 10 selected somaclones in relation to donor parent in replicated trail and initial screening.

Oil constituents	Somaclone number										Donor parent
	SC1	SC2	SC3	SC4	SC5	SC6	SC7	SC8	SC9	SC10	
Citronellal	25.53AB[a] ± 1.05[b] (24.4)[c]	23.2BC[a] ± 1.65[b] (21.3)[c]	26.33A[a] ± 0.85[b] (27.2)[c]	15.47F[a] ± 1.25[b] (17.3)[c]	17.17EF[a] ± 1.65[b] (14.5)[c]	26.23A[a] ± 2.77[b] (22.3)[c]	24.03AB[a] ± 0.67[b] (24.5)[c]	21.07CD[a] ± 0.51[b] (19.4)[c]	18.53DE[a] ± 1.12[b] (20.4)[c]	25.77AB[a] ± 1.62[b] (25.1)[c]	15.3F[a] ± 1.87[b] (17.5)[c]
Geraniol	18.4B[a] ± 1.15[b] (19.5)[c]	22.4A[a] ± 0.95[b] (16.8)[c]	18.63B[a] ± 1.96[b] (14.5)[c]	9.73E[a] ± 0.68[b] (11.7)[c]	15.53C[a] ± 1.05[b] (12.6)[c]	20.37AB[a] ± 0.85[b] (15.6)[c]	12.63D[a] ± 0.85[b] (17.3)[c]	14.76CD[a] ± 2.70[b] (13.3)[c]	13.37CD[a] ± 2.52[b] (15.2)[c]	19.6B[a] ± 0.8[b] (13.4)[c]	9.47E[a] ± 0.96[b] (12.2)[c]

Mean ± SD, means followed by the same capital letters are not significantly different at $P \leq 0.05$ (Duncan, 1955). [a]Values obtained in replication trial. [b]Standard deviation. [c]Values obtained in initial screening.

donor parent and mean values of somaclones for all characters (except leaf breadth) were also higher signifying a high degree of heterogeneity of embryogenic culture derived somaclones. This higher degree of variation in in vitro raised somaclones in contrast to parent for almost all the characters considered is in conformity with the results of earlier workers for different aromatic and medicinal plants (Marhur et al., 1988; Nayak et al., 2003; Kukreja et al., 1991; Viehmannova et al., 2014). Level of significance (*t*-test) of mean data for all eight traits between the two groups of plants was also analyzed (Table 1) and significant differences were found for mean diameter of bush, number of tiller/clump, number of leaves/clump and oil content between two groups of plants. However, we did not encounter any morphological variations such as pigmented leaves, albinism among the somaclones as has been observed in the field grown somaclones of *C. winterianus* and *C. martinii* (Marhur et al., 1988; Patnaik et al., 1999).

The data of somaclones for all the eight characters were classified in to five equal classes (Fig. 2) and it becomes clear that the frequency distribution of the somaclones was more evenly spread in the case of leaf length and weight of 100 leaves. Whereas, the frequency distribution analysis exhibited an uneven spread for plant height, diameter of bush, number of tiller/clump, number of leaves/clump, leaf length, leaf breadth and oil content. Therefore, it was evident that a greater degree of variation could be generated for the former two traits as against the latter six.

In order to select superior somaclones and to have an estimate of the stability of the generated variations, performance of some somaclones was recorded and analyzed statistically in initial screening and in replicated trial. Primarily a total of 10 superior somaclones were selected on the basis of results of initial screening and replicated trial (RBD) and the results in terms of mean and their standard deviation of the first harvest of the trial are outlined in Tables 2 & 3. Differences between the different somaclones for the eight different agronomic traits such as plant height, diameter of bush, number of tiller/clump, number of leaves/clump, leaf length, leaf breadth, weight of 100 leaves and oil content were evident at 1% level of significance. Significant differences between the different somaclones and also with donor parent were also evidenced by Duncan's Multiple Range Test (DMRT). We also estimated the superiority of these somaclones in RBD trial by considering the performance of the donor parent as 100 (Table 4). To select these somaclones we gave more attention to the economically important characters like oil content and oil quality. It was noticed that these 10 somaclones retained relatively high quantity of oil ranging between 2.35% and 3.12% in initial screening against the 1.21% in donor parent and ranging from 2.33% to 3.22% in RBD trial against 1.15% in donor parent. Enhancement in oil content in somaclones ranged from 102% to 180% compared to the control. On the basis of oil content of the 10 somaclones four somaclones (SC1, SC3, SC6 and SC10) were selected as they maintained their superiority over the parent line with >1.5 fold high oil content in RBD trial. Considering oil content SC2 was also selected as it has shown 146% enhancement in oil content compared to the control plant. The oil content in the rest of the somaclones (SC4, SC5, SC7, SC8 and SC9) was also high (>1 fold and <1.5 fold) in contrast to the parent.

The percent composition of two major constituents of oil (citronellal and geraniol) of the 10 selected somaclones was analyzed (Table 5) which have executed better to the parent plant in terms of oil content. There were significant variations in constituents for citronellal and geraniol among the 10 selected somaclones and also compared to the donor parent. It was observed that these selected somaclones retained the oil quality containing relatively a high amount of citronellal (ranging between 14.5% and 25.1% in initial screening against 17.5% in donor line and ranging from 15.47 to 26.33% in RBD trial against 15.3% in parent plant) and geraniol (ranging between 11.7% and 19.5% in initial screening against 12.2% in parent line and ranging from 9.73 to 22.4% in RBD trial against 9.47% in donor line). Such type of enhancement in geraniol and citronellal content was also reported in somaclones of *C. martinii*, citronella Java and Jamrosa (Marhur et al., 1988; Patnaik et al., 1999;

Table 6
Stability of selected traits of 5 improved somaclones and donor parent (control) in four clonal generations during two successive years. Mean ± SD, means followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan, 1955).

Clonal generation	Traits	Somaclone number					Control (M ± SD)
		SC1 (M ± SD)	SC2 (M ± SD)	SC3 (M ± SD)	SC6 (M ± SD)	SC10 (M ± SD)	
1st	Oil content (%)	$3.06^{} \pm 0.10$ (2.95–3.14)	$2.81^{b} \pm 0.12$ (2.7–2.94)	$3.21^{a} \pm 0.12$ (3.08–3.33)	$3.03^{a} \pm 0.1$ (2.87–3.14)	$3.02^{a} \pm 0.09$ (2.95–3.10)	$1.15^{c} \pm 0.05$ (1.10–1.21)
	Geraniol content (%)	$18.40^{b} \pm 1.15$ (17.2–19.5)	$22.40^{a} \pm 0.95$ (21.4–23.3)	$18.63^{b} \pm 1.95$ (16.6–20.5)	$20.10^{b} \pm 0.95$ (19.5–21.2)	$19.60^{b} \pm 0.80$ (18.8–20.4)	$9.23^{c} \pm 1.09$ (8.6–10.5)
	Citronellal content (%)	$25.80^{a} \pm 1.13$ (24.5–26.6)	$23.2^{a} \pm 1.65$ (21.5–24.8)	$26.40^{a} \pm 0.85$ (25.5–27.2)	$26.56^{a} \pm 2.89$ (23.3–28.8)	$25.83^{a} \pm 1.60$ (24.3–27.5)	$14.86^{b} \pm 1.81$ (13.5–17)
2nd	Oil content (%)	$3.01^{b} \pm 0.07$ (2.96–3.10)	$2.84^{b} \pm 0.06$ (2.78–2.90)	$3.23^{a} \pm 0.09$ (3.12–3.30)	$3.02^{b} \pm 0.17$ (2.83–3.14)	$2.93^{b} \pm 0.04$ (2.90–2.99)	$1.13^{c} \pm 0.07$ (1.05–1.20)
	Geraniol content (%)	$18.06^{b} \pm 1.20$ (16.8–19.2)	$23.13^{a} \pm 0.86$ (22.2–23.9)	$18.86^{b} \pm 3.09$ (15.5–21.6)	$19.23^{b} \pm 1.35$ (17.8–20.5)	$20.63^{ab} \pm 1.17$ (19.3–21.5)	$9.73^{c} \pm 1.17$ (7.7–11.00)
	Citronellal content (%)	$25.16^{ab} \pm 1.66$ (23.3–26.5)	$23.10^{b} \pm 1.22$ (22.2–24.5)	$26.83^{a} \pm 1.52$ (25.5–28.5)	$25.86^{ab} \pm 2.08$ (23.6–27.7)	$25.40^{ab} \pm 1.90$ (23.3–27)	$13.10^{c} \pm 2.11$ (11.5–15.5)
3rd	Oil content (%)	$3.00^{a} \pm 0.08$ (2.94–3.10)	$2.87^{b} \pm 0.05$ (2.81–2.91)	$3.26^{b} \pm 0.13$ (3.11–3.36)	$2.96^{b} \pm 0.11$ (2.90–3.10)	$2.99^{b} \pm 0.08$ (2.92–3.08)	$1.16^{c} \pm 0.05$ (1.11–1.21)
	Geraniol content (%)	$18.70^{b} \pm 1.25$ (17.5–20)	$21.86^{a} \pm 1.32$ (20.7–23.3)	$18.63^{b} \pm 2.31$ (16.2–20.8)	$19.96^{ab} \pm 1.81$ (18.5–22)	$19.53^{ab} \pm 1.74$ (17.6–21)	$8.89^{c} \pm 0.97$ (8.1–9.99)
	Citronellal content (%)	$22.93^{a} \pm 4.75$ (25–27.5)	$23.56^{a} \pm 2.05$ (21.5–25.6)	$26.00^{a} \pm 1.51$ (24.3–27.2)	$25.43^{a} \pm 2.61$ (22.5–27.5)	$26.20^{a} \pm 1.35$ (24.8–27.5)	$14.46^{b} \pm 2.65$ (12.6–17.5)
4th	Oil content (%)	$3.05^{a} \pm 1.10$ (2.97–3.12)	$2.85^{a} \pm 0.12$ (2.71–2.96)	$3.21^{a} \pm 0.13$ (3.06–3.31)	$3.01^{a} \pm 0.15$ (2.85–3.16)	$3.04^{a} \pm 0.09$ (2.94–3.12)	$1.41^{b} \pm 0.50$ 1.08–1.19)
	Geraniol content (%)	$17.66^{a} \pm 1.10$ (16.5–18.7)	$19.63^{a} \pm 5.73$ (21.5–24.2)	$18.46^{a} \pm 2.28$ (16–20.5)	$20.26^{a} \pm 1.67$ (19.2–22.2)	$19.40^{a} \pm 1.01$ (18.5–20.5)	$9.46^{b} \pm 0.95$ (8.5–10.4)
	Citronellal content (%)	$25.63^{ab} \pm 0.77$ (24.5–26)	$23.20^{b} \pm 1.83$ (21.2–24.8)	$26.53^{a} \pm 1.25$ (25.3–27.8)	$26.60^{a} \pm 2.33$ (24–28.5)	$25.30^{ab} \pm 1.66$ (23.5–26.6)	$14.93^{c} \pm 1.77$ (13–16.5)

Nayak et al., 2003). SC6 was selected on the basis of its better oil quality as it contained higher amount of geraniol (20.37%) and citronellal (26.23%) which also showed superiority in oil content. Somaclones Such as SC1, SC2, SC3 and SC10 also maintained their oil quality with respect to percent composition of citronellal and geraniol. SC7 contained a higher amount of citronellal (24.03%) with a lower geraniol and oil content. It was also observed that in some somaclones the oil quality is not directly proportional to the high oil quantity. Similar observations were also reported in in vitro regenerated plants of Java citronella and *Mentha* (Marhur et al., 1988; Kukreja et al., 1992). However, in the present investigation, the majority of the somaclones revealed more favorable variation in oil content and oil quality in replicated trial helping to select the somaclones with high oil quantity together with oil quality.

The 10 superior somaclones of *C. winterianus* which retained improved oil content and quality in initial screening and replicated trial were assessed for their stability in clonal propagation in the field in subsequent years. The quality of oil (geraniol and citronellal) in SC4 decreases from first clonal generation and this decrease becomes dramatic from third clonal generation showing total geraniol and citronellal content less than the control. Somaclone No. 7 and 8 maintained >1 fold oil yield than control up to two clonal propagation failed to maintained this trait from third clonal propagations onwards and became comparable to the control. The somaclone line 5 and 9 also maintaned their superiority in oil content (more than 1 fold increase) compared to donor parent in successive clonal generations but their oil quality (citronellal and geraniol percentage) is decreased gradually in successive clonal propagations. From the 10 selected somaclones rest five superior somaclonal lines namely SC1, SC2, SC3, SC6 and SC10 were further propagated and they showed relative stability for the main selected traits such as high oil content, high geraniol and citronellal content up to 4th clonal propagation of the 2nd year, 2008 (Table 6).

Table 7
Details of RAPD analysis of variant somaclones.

Sl. no	Primer	Sequence of the primer (5′-3′)	No. of amplified bands in						No. of polymorphic bands between													
			Mother plants (M)	Variant somaclones					Mother and somaclones					Somaclones								
				SC1	SC2	SC3	SC6	SC10	M & SC1	M & SC2	M & SC3	M & SC6	M & SC10	SC1 & SC2	SC1 & SC3	SC1 & SC6	SC1 & SC10	SC2 & SC3	SC2 & SC6	SC2 & SC10	SC3 & SC6	SC3 & SC10	SC6 & SC10
1	MS10G3	GTCCTTAGCG	3	2	3	2	2	1	1	0	1	1	2	1	0	0	1	1	1	2	0	1	1
2	MS10G4	TGCGCGATCG	5	5	4	5	5	4	0	3	0	0	1	3	0	0	1	3	3	2	0	1	1
3	MS10G15	GTCCTACTCG	8	6	6	6	6	8	2	2	2	2	0	0	0	0	2	0	0	2	0	2	2
4	MS10G5	AACGTACGCG	12	11	12	7	7	13	3	4	5	5	3	7	6	6	4	7	7	3	0	6	6
5	MS10G7	CTATCGCCGC	4	4	5	5	6	2	2	3	3	2	2	3	3	4	2	0	1	3	1	3	4
6	MS10G11	GCACGCCGGA	6	6	6	4	4	6	0	0	4	2	0	0	4	2	0	4	2	0	2	4	2
7	MS10G17	GCGAATTCCG	4	3	2	3	3	4	1	2	1	1	0	1	2	0	1	1	1	2	2	1	1
8	MS10G1	AAATCGGAGC	5	6	4	6	5	5	1	1	0	0	0	2	0	1	1	2	1	1	1	1	0
9	MS10G8	CGGGATCCGC	6	6	6	6	6	6	0	0	2	4	0	0	2	4	0	2	4	0	4	2	4
10	MS10G10	CACCCTGCGC	7	2	7	8	7	8	7	0	1	0	3	7	8	7	8	1	0	3	1	2	3
Total			60	51	55	52	51	57	17	15	19	15	11	24	25	24	20	21	20	18	11	23	24

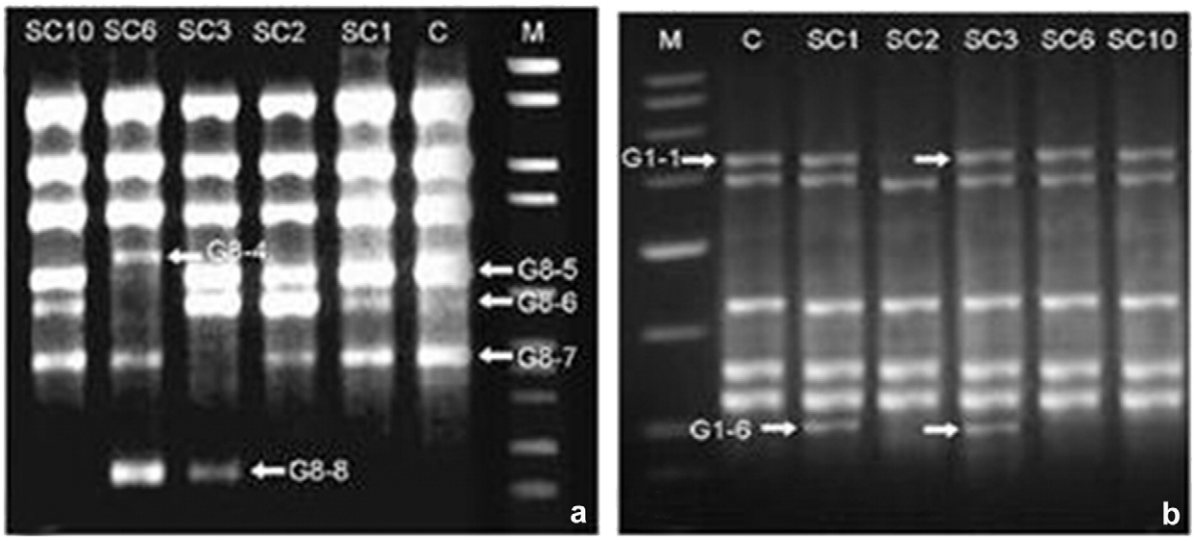

Fig. 3. RAPD profile of control plant and five somaclonal variants of *C. winterianus* generated by primer (a) MS10G8 and primer (b) MS10G1. C = mother plant (control), SC1 = somaclonal variant no. 1, SC2 = somaclonal variant no. 2, SC3 = somaclonal variant no. 3, SC6 = somaclonal variant no. 6 and SC10 = somaclonal variant no. 10, and M = molecular marker, λ DNA digested with *EcoRI* and *HindIII*, arrow indicates polymorphic bands generated.

3.3. RAPD analysis

These five somaclones are subjected to RAPD using 18 arbitrary primers and the results were compared to the control. Out of the 18 primers used, 10 primers revealed polymorphism showing distinctly different banding patterns in the five improved somaclones, which were equally prominent in their differences from the control (Table 7 & Fig. 3). The sequence of the primers and the specific amplified fragments of the DNA, which were generated against the primers, are presented in Table 8. In general 1–13 amplified fragments were scored depending upon the primers. The number of polymorphic fragments between the mother and five different somaclones was also calculated. A maximum of 19 polymorphic bands were obtained between the mother and SC3, while a minimum number of polymorphic fragment (11) were calculated between the mother and SC10 using the 10 RAPD primers. A number of polymorphic fragment between the somaclones were maximum (25) between the SC1 and SC3 and minimum (11) between SC3 and SC6.

In the present experiment, ten out of eighteen primers gave 3–15 distinct bands per primer, ranging in molecular size from 60 to 5200 base pairs. A maximum of 15 loci were amplified with primer MS10G5, while a minimum of 3 loci were recorded with primer MS10G3 with an average of 7.4 bands per RAPD primer (Table 8). Out of the 74 amplified bands 30 (40.54%) were monomorphic and 44 (59.46%) were polymorphic in nature. The variation in banding pattern indicates a high degree of polymorphism between the somaclones and with the mother. The number of polymorphic bands ranged 2–10, with an average of 4.4 polymorphic bands per primer. Primer MS10G10 generated 90% polymorphic bands, while MS10G15 produced 25% polymorphic bands. The average PIC value of the primers was 0.21, ranging from 0.111 (MS10G15) to 0.285 (MS10G7) and the average marker index was calculated as 0.71, ranging from 0.185 (MS10G15) to 1.84 (MS10G5). Out of the 44 polymorphic bands, 30 were parental bands (Table 9) which were missing from some somaclonal progenies and 14 were novel non-parental bands (Table 10) which were detected only in somaclones (SC1, SC2, SC3, SC6, SC10). A maximum number of three polymorphic non-parental bands were generated by the primer MS10G5 (G5-3, G5-7, G5-10), MS10G7 (G7-1, G7-2, G7-7) and MS10G10 (G10-2, G10-3, G10-8), whereas a minimum number of one non-parental polymorphic band were generated by the primer MS10G4 (G4-4), MS10G11 (G11-4), MS10G1 (G1-6) (Table 10 & Fig. 3b). G8-4 and G8-8 are the two non parental polymorphic bands produced by the primer MS10G8 (Table 10 & Fig. 3a). Out of the 30 parental polymorphic bands the highest number of 7 polymorphic bands were generated by the primer MS10G5 (G5-2, G5-4, G5-6, G5-8, G5-11, G5-13, G5-14) (Table 9) and the lowest number of 2 were produced by the primer MS10G3 (G3-2, G3-3), MS10G4 (G4-5, G4-6), MS10G2 (G2-1, G2-2), MS10G7 (G7-3, G7-4) and MS10G9 (G9-1, G9-4) (Table 9). Evidently, SC3 had accumulated maximum genetic change as represented by the presence of 6-non-parental and the absence of 14 parental bands, while SC10 had undergone minimum genetic change containing 4 non-parental bands and absence of seven parental bands.

Table 8
List of RAPD primers tested for their efficiency in generating polymorphism in mother plant and somaclones.

Sl. no.	Primer designation	Primer sequence	Fragment length (bp)	Total no. of bands	Monomorphic bands	Polymorphic bands	Polymorphic bands (%)	PIC values (0.0–0.5)	Marker index (MI)
1	MS10G3	GTCCTTAGCG	250–370	3	1	2	66.67	.241	.35
2	MS10G4	TGCGCGATCG	250–900	6	3	3	50	.166	.388
3	MS10G15	GTCCTACTCG	190–5200	8	6	2	25	.111	.185
4	MS10G5	AACGTACGCG	200–3200	15	5	10	66.67	.266	1.84
5	MS10G7	CTATCGCCGC	100–2800	7	2	5	71.43	.285	.876
6	MS10G11	GCACGCCGGA	200–1600	7	3	4	57.14	.206	.626
7	MS10G17	GCGAATTCCG	290–990	4	2	2	50	.236	.374
8	MS10G1	AAATCGGAGC	300–1400	6	4	2	33.33	.12	.205
9	MS10G8	CGGGATCCGC	150–2900	8	3	5	62.50	.16	.60
10	MS10G10	CACCCTGCGC	60–850	10	1	9	90	.283	1.66
		Total		74	30	44			

Table 9
Presence of polymorphic parental RAPD bands in variant somaclones of *Cymbopogon winterianus*.

Polymorphic bands	Mother	Somaclone number				
		SC1	SC2	SC3	SC6	SC10
G3-2	+	+	+	+	+	−
G3-3	+	−	+	−	−	−
G4-5	+	+	−	+	+	−
G4-6	+	+	−	+	+	+
G2-1	+	−	−	−	−	+
G2-2	+	−	−	−	−	+
G5-2	+	+	−	−	−	−
G5-4	+	+	+	−	−	+
G5-6	+	−	+	−	−	+
G5-8	+	−	+	+	+	+
G5-11	+	+	−	+	+	+
G5-13	+	+	+	−	−	+
G5-14	+	+	+	−	−	+
G7-3	+	−	−	−	+	−
G7-4	+	+	+	+	+	−
G11-3	+	+	+	−	−	+
G11-5	+	+	+	−	+	+
G11-6	+	+	+	−	−	+
G9-1	+	+	−	−	+	+
G9-4	+	−	−	+	−	+
G1-1	+	+	−	+	+	+
G8-5	+	+	+	+	−	+
G8-6	+	+	+	+	−	+
G8-7	+	+	+	−	+	+
G10-1	+	−	+	+	+	+
G10-4	+	−	+	+	+	−
G10-5	+	−	+	+	+	+
G10-6	+	−	+	+	+	+
G10-7	+	−	+	+	+	+
G10-9	+	−	+	+	+	+

Similarity index values were calculated using the RAPD data for the mother plant and the five improved somaclones (Table 11). Similarity index ranged from 0.66 to 0.85 with an average of 0.74 indicating a close genetic relationship within the somaclones as well as with the mother plant. Similarity index between the mother and somaclones was ranged between 0.73 and 0.85 which revealed a high degree of relatedness of somaclones (SC1, SC3, SC3, SC6 and SC10) to the parent. The genetic similarity between mother and SC10 was sufficiently high showing a maximum similarity coefficient value of 0.85, whereas SC3 had undergone the highest degree of genetic change from the parent which was evidenced by the lowest value for similarity coefficient (0.73) between them. Likewise, SC1, SC2 and SC6 showed a moderate degree of similarity with the parent carrying a similarity coefficient of 0.77, 0.80 and 0.77 respectively. Similarity index among the somaclones

Table 10
Presence of non-parental polymorphic RAPD bands in variant somaclones of *Cymbopogon winterianus*.

Polymorphic bands	Mother	Somaclone number				
		SC1	SC2	SC3	SC6	SC10
G4-4	−	−	+	−	−	−
G5-3	−	+	−	−	−	+
G5-7	−	−	+	−	−	−
G5-10	−	−	+	−	−	+
G7-1	−	−	+	+	+	−
G7-2	−	−	+	+	+	−
G7-7	−	+	−	−	−	−
G11-4	−	−	−	+	−	−
G1-6	−	+	−	+	−	−
G8-4	−	−	−	−	+	−
G8-8	−	−	−	+	+	−
G10-2	−	−	−	−	−	+
G10-3	−	−	−	+	−	+
G10-8	−	+	−	−	−	−

Table 11
Jaccard's similarity coefficient of mother plant and five somaclones.

	Mother	SC1	SC2	SC3	SC6	SC10
Mother	1.0000					
SC1	0.7703	1.0000				
SC2	0.7973	0.6757	1.0000			
SC3	0.7297	0.6622	0.7162	1.0000		
SC6	0.7703	0.6757	0.7297	0.8514	1.0000	
SC10	0.8514	0.7297	0.7568	0.6892	0.6757	1.0000

also varied from 0.66 to 0.85 expressing high degree of genetic relationship. The highest degree of genetic dissimilarity among the five improved somaclones was found between the SC1 and SC3 and had the lowest similarity value of 0.66, while the lowest degree of genetic dissimilarity or highest degree of genetic similarity was observed in between SC3 and SC6 bearing highest value (0.85) of similarity.

In *Cymbopogon*, RAPDs have been used efficiently to study genetic diversity among elite varieties (Sangwan et al., 2001, 2003; Shasany et al., 2000), to identify somaclonal variants (Nayak et al., 2003), to establish species relationships and to study the genetic polymorphism of somatic embryo derived plantlets (Bhattacharya et al., 2008). In our study, the PCR-based molecular marker technique known as RAPD has been shown to be efficient in the analysis of culture derived regenerated plants i.e., variants in order to find out the changes that had occurred at the DNA level during tissue culture procedure and the results pointed out that genetic changes have come about in somaclones. This result is in conformance with determination of genetic polymorphism of somaclones by RAPD technique in *Cymbopogon* (Patnaik and Debata, 2001; Nayak et al., 2003) and other plants also such as, peach (Hashmi et al., 1997), rice (Yang et al., 1999), maize (Osipova et al., 2001), tomato (Soniya et al., 2001), *Chrysanthemum* (Martín et al., 2002), sugarcane (Tawar et al., 2008), olive tree (Peyvandi et al., 2010), and banana (Abdellatif et al., 2012).

The polymorphism in RAPD pattern may be due to different causes including loss/gain of primer annealing, due to point mutation or by insertion or deletion of sequences or transposable elements. Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions have induced varied amount of genetic changes in different regenerated plants (Soniya et al., 2001). Some of the non-parental bands are common in different somaclones which indicates the occurrence of identical genetic changes and this can be explained by the origin of somaclones from same embryogenic callus tissue. The main type of variation at the nucleotide sequence level was nucleotide substitution, mostly transition while transversions were rare (Ngezahayo et al., 2007).

Though many authors reported that plants regenerated through somatic embryogenesis exhibit genetic uniformity and integrity (Jayanthi and Mandal, 2001; Mallón et al., 2010; Rai et al., 2012), several reports substantiate the presence of genetic modification in somatic embryo derived plants (Rani et al., 1995; Jin et al., 2008; Prado et al., 2010; Abdellatif et al., 2012; Viehmannova et al., 2014). Moreover, callus culture is an important source of somaclonal variation which was first confirmed by Larkin and Scowcroft (1981) and in our study we have regenerated plants through somatic embryogenesis with intermittent callus phase which in turn maximizes the chance of genetic change among regenerants. A disorganized growth phase in tissue culture, the use of growth regulators, the number and duration of subculture, stress and the genotype are all factors that enhance the somaclonal variation (Bairu et al., 2011). Therefore, indirect method of plant regeneration through callus culture is preferred for breeding when the objective is to get genetic diversity for introduction of novel traits and these variations can be used for selection of superior clones in such a vegetative propagated aromatic plant where alternative breeding approaches are limited due to extremely poor seed setting and low germination percentage. However, the extremely high rate of variations detected within

somaclonal variants could be attributed to the pre-existing heterogeneity due to out-breeding and chromosomal variation of seed grown natural population used as explants source. Another reason for obtaining such genetic variations is the repeated and extended sub-culturing in 2, 4-D containing medium used for the establishment of embryogenic callus from rhizome explants (Raimondi et al., 2001; Nayak et al., 2003; Saker et al., 2000). To this variation must be added the usual alteration induced by the stress conditions of in vitro culture (Martín et al., 2002).

So the results of this study suggest that considerable somaclonal variations occur in regenerants derived from an in vitro culture system (somatic embryogenesis). The plants regenerated exhibit variation at the agromorphological, chemical and molecular levels. Moreover, it should be possible to discern some of the somaclonal variation using agromorphological and genetically characterized RAPD markers. Thus, molecular changes can reflect stable changes in the genome that, introducing more variation in *C. winterianus* germplasm, could be valuable in program designed to improve characteristics of this aromatic grass.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2014.10.010.

References

Abdellatif, M.F., Hegazy, A.E., Aboshama, H.M., Emara, H.A., El-Shahed, A.A., 2012. Morphological and molecular characterization of somaclonal variations in tissue culture-derived banana plants. Journal of Genetic Engineering and Biotechnology 10, 47–53.

Aruingtyas, E.L., Widoretno, W., Idriyani, S., 2012. Somaclonal variation of soybean (Glysin max. L. Merr) stimulated by draught stress based on Random Amplified Polymorphic DNA (RAPDs). American Journal of Molecular Biology 2, 85–91.

Bairu, M.W., Aremu, A.O., Van Staden, J., 2011. Somaclonal variation in plants: causes and detection methods. Plant Growth Regulation 63, 147–173.

Bajaj, Y.P.S., 1990. Somaclonal variation—origin, induction, cryopreservation and implication in plant breeding. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry 11Somaclonal Variation in Crop Improvement. Springer-Verlag, Berlin, Heidelberg, New York, pp. 3–48.

Bhattacharya, S., Dey, T., Bandopadhyay, T.K., Ghosh, P.D., 2008. Genetic polymorphism analysis of somatic embryo-derived plantlets of *Cymbopogon flexuosus* through RAPD assay. Plant Biotechnology Reports 2, 245–252.

Clevenger, J.F., 1928. Apparatus for determination of volatile oil. Journal of the American Pharmaceutical Association 17, 346.

Dey, T., Bhattacharya, S., Ghosh, P.D., 2010a. Somatic embryogenesis from rhizome explants of *Cymbopogon winterianus*. Biologia Plantarum 54 (2), 325–328.

Dey, T., Saha, S., Dhar, T.N., Adhikary, S., Ghosh, P.D., 2010b. Optimization and comparison of efficiency between two DNA isolation protocols in *Cymbopogon* species. General and Applied Plant Physiology 36 (3–4), 232–238.

Duncan, D.B., 1955. Multiple range and multiple F-test. Biometrics 11, 1–42.

Ford-Lloyd, B.V., Sabir, A., Newbury, H.J., Todd, C., Catty, J., 1992. Determination of genetic stability using isozymes and RFLPs in beet plants regenerated *in vitro*. Theoretical and Applied Genetics 84, 113–117.

Guenther, E., 1972. The Essential Oils vol. I. Robert E. KriegerPubl Co, New York, Huntington, pp. 316–319.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L., Hammerschlag, F., 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Reports 16, 624–627.

Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. California Agricultural Experiment Station, Circular 347.

Hussain, A., 1982. A quarter century of research on essential crops in India. Indian Perfumer 26, 17–28.

Jaccard, P., 1908. Nouvelles recherches sur la distribution florale. Bulletin de la SosiétéVaudoise Sciences Naturelles 44, 223–270.

Jain, S.M., 2001. Tissue culture derived variation in crop improvement. Euphytica 118, 153–166.

Jayanthi, M., Mandal, P.K., 2001. Plant regeneration through somatic embryogenesis and RAPD analysis of regenerated plants of *Tylophora indica* (Burm. f. Merrill). In Vitro Cellular & Developmental Biology—Plant 37, 576–580.

Jin, S., Mushke, R., Zhu, H., Tu, L., Lin, Z., Zhang, Y., Zhang, X., 2008. Detection of somaclonal variation in cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Reports 27, 1303–1316.

Kukreja, A.K., Dhawan, O.P., Mathur, A.K., Ahuja, P.S., Mandal, S., 1991. Screening and evaluation of agronomically useful somaclonal variation in Japanese mint (*Mentha arvensis* L.). Euphytica 53, 183–191.

Kukreja, A.K., Dhawan, O.P., Ahuja, P.S., Sharma, S., Mathur, A.K., 1992. Genetic improvements of mints: on the qualitative traits of essential oil of in vitro derived clones of Japanese mint (*Mentha arvensis* var. *piperascens* Holms). Journal of Essential Oil Research 4, 623–629.

Larkin, P.J., Scowcroft, W.R., 1981. Somaclonal variations—a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60, 197–214.

Lavania, U.C., 1988. Karyomorphological observations in *Cymbopogon* sp. Cytologia 53, 517–524.

Mallón, R., Oubina, J.R., Gonlàlez, M.L., 2010. In vitro propagation of the endangered plant *Centaurea ultreiae*: assessment of genetic stability by cytological studies, flow cytometry and RAPD analysis. Plant Cell, Tissue and Organ Culture 103, 31–39.

Marhur, A.K., Ahuja, P.S., Pandey, B., Kukreja, A.K., Mandal, S., 1988. Screening and evaluation of somaclonal variation for quantitative and qualitative traits in an aromatic grass *C. winterianus* Jowitt. Plant Breeding 101, 321–334.

Martín, C., Uberhuaga, E., Pérez, C., 2002. Application of RAPD markers in the characterization of *Chrysanthemum* varieties and the assessment of somaclonal variation. Euphytica 127, 247–253.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15, 473–497.

Nayak, S., Debata, B.K., Srivastava, V.K., Sangwan, N.S., 2003. Evaluation of agronomically useful somaclonal variants in Jamrosa (a hybrid Cymbopogon) and detection of genetic changes through RAPD. Plant Science 164, 1029–1035.

Ngezahayo, F., Dong, Y., Liu, B., 2007. Somaclonal variation at the nucleotide sequence level in rice (*Oryza sativa* L.) as revealed by RAPD and ISSR markers and by pairwise sequence analysis. Journal of Applied Genetics 48 (4), 329–336.

Osipova, E.S., Kokaeva, Z.G., Troitskij, A.V., Dolgikh, Y.I., Shamina, Z.B., Gotimskij, S.A., 2001. RAPD analysis of maize somaclones. Genetika-Moskva 37 (1), 91–96.

Patnaik, J., Debata, B.K., 2001. RAPD analysis of variant somaclones of palmarosa (*Cymbopogon martinii* var. *motia*). In: Sahoo, S., Ramesh, D.B., Rao, Y.R., Debata, B.K., Misra, V. (Eds.), Conservation and Utilization of Medicinal and Aromatic Plants. RRL, Bhubaneswar, Allied Publishers Ltd, New Delhi, pp. 296–301.

Patnaik, J., Sahoo, S., Debata, B.K., 1999. Somaclonal variation in cell suspension culture-derived regenerants of *Cymbopogon matinii* (Roxb.) Wats var. *motia*. Plant Breeding 118, 351–354.

Peschke, V.M., Phillips, R.L., 1992. Genetic implications of somaclonal variation in plants. Advances in Genetics 30, 41–75.

Peyvandi, M., Farahzadi, H.N., Arbabian, S., Noormohammadi, Hosseini-Mazinami, 2010. Somaclonal variation among somatic embryo derived plants of *Olea europaea* L "cv. Kroneiki". Journal of Sciences, Islamic Republic of Iran 21 (1), 7–14.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) makers for germplasm analysis. Molecular Breeding 2, 225–238.

Prado, M.J., Rodriguez, E., Rey, L., Gonlàlez, M.V., Santos, C., Rey, M., 2010. Determination of somaclonal variants in somatic embryogenesis regenerated plants of *Vitis vinifera* by flow cytometry and microsatellite markers. Plant Cell, Tissue and Organ Culture 103, 43–59.

Rai, M.K., Phulwaria, M., Harish, Gupta, A.K., Shekhawat, N.S., Jaiswal, U., 2012. Genetic homogeneity of guava plant derived from somatic embryogenesis using SSR and ISSR marker. Plant Cell, Tissue and Organ Culture 111, 259–264.

Raimondi, J.P., Masuelli, R.W., Camadro, E.L., 2001. Assessment of somaclonal variation in asparagus by RAPD fingerprinting and cytogenetic analysis. Scientia Horticulturae 90, 19–29.

Rani, V., Parida, A., Raina, S.N., 1995. Randomly amplified polymorphic DNA (RAPD) marker for genetic analysis in micropropagated plants of *Populus deltoids* marsh. Plant Cell Reports 14, 450–462.

Rohlf, F.J., 2000. NTSYS pc-Numerical Taxonomy and Multivariate Analysis System, Version-2. 1. Applied Biostatistics Inc., New York, USA.

Saker, M.M., Bekheet, S.A., Taha, H.S., Fahmy, A.S., Moursy, H.A., 2000. Detection of somaclonal variation in tissue culture derived date palm plants using isoenzyme analysis and RAPD fingerprints. Biologia Plantarum 43 (3), 347–351.

Sangwan, N.S., Yadav, U., Sangwan, R.S., 2001. Molecular analysis of genetic diversity in elite Indian cultivars of essential oil trade types of aromatic grasses (*Cymbopogon* species). Plant Cell Reports 20, 437–444.

Sangwan, N.S., Jadav, U., Sangwan, R.S., 2003. Genetic diversity among elite varieties of the aromatic grasses, *Cymbopogon martinii*. Euphytica 130, 117–130.

Shasany, A.K., Lal, R.K., Patra, N.K., Darokar, M.P., Garg, A., Kumar, S., Khanuja, S.P.S., 2000. Phenotypic and RAPD diversity among *Cymbopogon winterianus* Jowitt accessions in relation to *Cymbopogon nardus* Rendle. Genetic Resources and Crop Evolution 47, 553–559.

Soniya, E.V., Banerjee, N.S., Das, M.R., 2001. Genetic analysis of somaclonal variation among callus-derived plants of tomato. Current Science 80, 1213–1215.

Sreenath, H.L., Jagadishchandra, K.S., 1991. *Cymbopogon spreng* (aromatic grasses) in vitro culture, regeneration and production of essential oils. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and ForestryMedicinal and Aromatic Plants III vol. 15. Springer-Verlag, Berlin-Heidelberg, New York, pp. 211–236.

Tawar, P.N., Sawant, R.A., Dalvi, S.G., Nikam, A.A., Tawar, P.G., Devarumath, R.M., 2008. An assessment of somaclonal variation in micropropagated plants of sugarcane by RAPD markers. Sugar Technology 10 (2), 124–127.

Viehmannova, I., Bortlova, Z., Vitamvas, J., Cepkova, P.H., Eliasova, K., Svobodova, E., Travnickova, M., 2014. Assessment of somaclonal variation in somatic embryo derived plants of Yacon [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Robinson] using inter simple sequence repeat analysis and flow cytometry. Electronic Journal of Biotechnology http://dx.doi.org/10.1016/j.ejbt.2013.12.011.

Yang, H., Tabei, Y., Kamada, H., Kayano, T., Takaiwa, F., 1999. Detection of somaclonal variation in cultured rice cells using digoxigenin-based random amplified polymorphic DNA. Plant Cell Rep 18, 520–526.