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In vitro propagation of the elite species plant *Pluchea lanceolata:* Assessment of genetic stability by random amplified polymorphic DNA (RAPD) analysis

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An effective *in vitro* regeneration protocol was developed from nodal segment of *Pluchea lanceolata* (DC.) Oliver. & Hiern, a medicinally important plant used in ayurvedic system of medicine for curing diseases similar to rheumatoid arthritis. Nodal segments were cultured in MS medium supplemented with auxin and cytokinin and their combinations. The objective was to produce genetically identical plants, via multiple shoot induction from nodal segment. The culture medium consisted of Murashige and Skoog medium supplemented with one of 3 cytokinins [6-benzyladenine (BAP), Kinetin (Kn) and thidiazuron (TDZ)] at each of six different concentrations for shoot multiplication. The highest multiplication rate (24.57 shoots per explant) was obtained in the medium enriched with NAA. Shoots were successfully rooted in the half strength MS medium containing 0.1 µM NAA. *In vitro* produced plants were transferred to sterilized garden soil: compost (1:1) and then transferred to green house for hardening. Genetic stability of mother plant and the regenerants produced *in vitro* was assessed by random amplified polymorphic DNA (RAPD). In randomly selected plant material (mother plant) and its regenerants, 87 scorable bands were generated by four different primers, showing monomorphism with the mother plant. Thus, molecular analysis reveals that the micropropagation system described is a reliable method for propagation of *P. lanceolata*.

Key words: Conservation, genetic fidelity, micropropagation, RAPD, TDZ.

INTRODUCTION

Pluchea lanceolata (DC.) belongs to the family of Asteraceae. It is perennial, native, under shrub. There are 11 species in the genus but nine have been abundantly used in traditional uses (GRIN, 2011). *P.*

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Abbreviations: MS, Murashige and Skoog medium; **TDZ,** 1- phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; **RAPD,** random amplified polymorphic DNA; **MP,** mother plant; **PCR,** polymerase chain reaction; **CTAB,** cetyltrimethylammonium bromide; **EDTA,** ethylenediaminetetraacetic acid; **BAP,** 6-benzyl aminopurine; **Kn,** Kinetin; **BM,** Basal culture medium; **NAA,** Naphthalenacetic acid; **IBA,** Indole butyric acid; **IAA,** Indole acetic acid; **PGR's,** Plant growth regulators.

lanceolata used in inflammation, bronchitis, psoriasis, cough and piles. It is also used as antipyretic, analgesic, laxative and uterine relaxant (Dwivedi et al., 1949; Nadkarni, 1954; Jadhav and Bhutani, 2005). In anemia and general debility it is salutary as a general tonic (Choudhary, 2012). *P. lanceolata* also exhibits anticancereous property and suppresses oxidative damage in Wistar rats (Jahangir and Sultana, 2006). *P. lanceolata* shows the immunosuppressive inhibition of Th1 cytokines (Bhagwat et al., 2010). Due to over collection and unscrupulous extraction of plants from the wild population for medicine and trade complexed with poor seed viability are the major factors for the continuous decline of its natural population in the country and the species has become vulnerable to extinction (Singh, 2004) and that is why the plant is listed in priority species by ministry health and family welfare, Govt. of India.

The plant is propagated from root but this method is time consuming and result in limited number of plants. Hence, development of an *in vitro* method for rapid micropropagation multiplication of *P. lanceolata* is warranted for providing uniform raw material for medicinal uses as well as its conservation. Production of genetically identical clones from axillary buds is achieved by various PGR's effectively. But cell mutations and genetic instability can happen because of many reasons such as the type of media used, PGR's and its concentration, the type of explant and number of subculture cycles (Bairu et al., 2011). So, it becomes necessary to check the genetic stability of the *in vitro* produced plants. In the present study, we aimed on (a) An efficient system of *in vitro* propagation from nodal segment of species and (b) Evaluation of genetic fidelity of *in vitro* regenerated plants.

MATERIALS AND METHODS

Plant material

Excised nodal segment from fresh twig were washed thoroughly under the running tap water for 30 min to remove the dust particles followed by treatment with 0.1% (w/v) Bavistin for 15 min along with 2% Tween-20 (v/v) for 10 min to remove the adhering particles and rinsed with sterilized distilled water. Explants were surface sterilized with 0.1% (w/v) $HgCl_2$ (Qualigens, India) for 60 s. This step was followed by rinsing 5 to 6 times with sterilized distilled water to remove the traces of sterilizant.

Culture media

The sterilized nodal explants were inoculated on basal culture medium (BM) consisting of MS medium (Murashige and Skoog, 1962), sucrose (20 gL^{-1}) and agar (8 gL^{-1}) with various concentration of PGR's viz., BAP (1.38 to 13.85 µM), Kn (1.45 to 14.5 µM) and TDZ (1.41 to 14.15 µM) alone or in combination. The pH of the medium was adjusted 5.8 before autoclaving for 20 min at 121˚C. The cultures were subcultured for 4 weeks until sufficient shoots were accumulated to enable shoot multiplication optimisation experiments.

Growth conditions for multiple shoot induction

Explants were inoculated with 50 ml of BM with different concentration of BAP, TDZ and Kn in culture tubes. The cytokinin treatment which gives the best result was tested in combination with NAA, IBA and IAA (0.1, 0.5 and 1.0 µM) for shoot multiplication. Cultures were incubated at 25±2˚C with a 16 h photoperiod (2000 to 2500 lux) with 40 W cool white fluorescent tubes. For rooting, the *in vitro* shoots were cultured on the inducing medium consisting of half strength MS with NAA, IAA and IBA for 4 weeks of culture. Then, *in vitro* rooted plantlets were transferred to a mixture containing sterilized garden soil and compost in 1:1 ratio and maintained in green house condition under natural light. After 4 weeks, acclimatized plants were transferred to earthen pots.

Statistical analysis

Three replicates (10 explants per replicate) were inoculated per treatment. Data were subjected to ANOVA (analysis of variance) treatment and means were determined by Duncan's multiple range test (DMRT) ($p \leq 0.05$) using SPSS 16.0 version, 2008.

DNA extraction and RAPD analysis

For genomic DNA isolation, young and fresh leaves were collected from the randomly selected cultures and also from field grown mother plants of *P. lanceolata* for comparison. Total genomic DNA was extracted from the frozen leaf material using standard protocol (Doyle and Doyle, 1990). RAPD profiles were produced through PCR amplifications. All the PCR reactions were carried out under optimized conditions in 0.2 ml polypropylene PCR tubes Bangalore Genei, India using Thermal cycler eppendrof. Each 25 µl reaction mixture contained 1x Taq buffer 100 mM Tris-Cl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% Gelatin, 2.5 mM $MgCl_2$, 100 µM dNTP mix Bangalore Genei, India and 50 ng of template DNA. All reactions were subjected to initial denaturation at 95°C for 5 min followed by 35 amplification cycles, each cycle consisting of 1 min at 43°C annealing step and 2 min at 72°C extension step with a final extension of 7 min at 72°C. Banding profiles generated by RAPD was compiled into a data binary matrix based on the presence (1) or absence (0) of the selected band. Only clear, unambiguous and reproducible bands amplified were considered for the scoring and data analysis. Data were analyzed using simqual route to generate Jaccard's similarity coefficient with NTSYS pc Version 2.02 k programme (Rohlf, 1998). Dendogram was prepared using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Sequential, Agglomerative, Hierarchical, and Nonoverlapping (SAHN) clustering for analysis of relationships.

RESULTS AND DISCUSSION

Multiple shoot induction

Establishment of the *in vitro* cultures in *P. lanceolata* posed considerable problem with contamination in primary cultures, and repeated subculture. The problem was overcome by treating the explants with 0.1% (w/v) Bavistin for 15 min and then with 0.1% $HgCl_2$ for 1 min. No organogenic changes were observed after 4 weeks, in the excised meristem cultured on BM devoid of PGR's. Presence of low and intermediate levels of TDZ produced maximum shoots per explant of the three cytokinins tested on *P. lanceolata*. There was a proportional

Table 1. Effect of various plant growth regulators on development of nodal explants in *P. lanceolata* after 8 weeks of culture.

Plant growth regulators (µM)						Mean number of shoots /explants	Mean shoot length
TDZ	BAP	Kn	IBA	IAA	NAA		
Control						1[a]	0
1.41						8.83 ± 0.59 [c]	2.00 ± 0.14[b]
2.83						16.48 ± 0.72[e]	2.60 ± 0.14[c]
5.66						12.90 ± 0.82[d]	1.84 ± 0.11[b]
8.49						7.93 ± 0.58[bc]	1.85 ± 0.11[b]
11.32						6.76 ± 0.89[c]	1.54 ± 0.20[b]
14.15						5.40 ± 0.47[b]	1.69 ± 0.15[b]
2.83			0.1			15.67 ± 1.05[cd]	3.28 ± 0.23[c]
2.83			0.5			14.97 ± 0.33[cd]	3.56 ± 0.32[c]
2.83			1			12.87 ± 0.88[c]	3.75 ± 0.24[c]
2.83				0.1		7.83 ± 0.15[b]	2.17 ± 0.32[b]
2.83				0.5		2.90 ± 0.24[a]	1.01 ± 0.43[a]
2.83				1		1.87 ± 0.24[a]	0.87 ± 0.58[a]
2.83					0.1	24.57 ± 0.37[e]	4.91 ± 0.27[d]
2.83					0.5	16.90 ± 0.46[d]	3.25 ± 0.28[c]
2.83					1	11.90 ± 0.38[c]	2.85 ± 0.31[bc]
	1.38					4.9 ± 0.66[a]	2.1 ± 0.05[a]
	2.77					6.33 ± 0.33[b]	2.3 ± 0.05[cd]
	5.54					8.66 ± 0.33[bc]	2.3 ± 0.0[d]
	8.31					10.08 ± 0.33[e]	2.5 ± 0.0[e]
	11.08					9.03 ± 0.47[bd]	2.2 ± 0.03[b]
	13.85					6.36 ± 0.53[a]	2.0 ± 0.03[a]
		1.45				3.66 ± 0.81[a]	2.0 ± 0.0[a]
		2.9				3.83 ± 0.88[a]	2.3 ± 0.0[d]
		5.8				5.03 ± 0.31[b]	2.5 ± 0.0[e]
		8.7				6.68 ± 0.34[cd]	2.5 ± 0.06[e]
		11.6				6.94 ± 0.35[cde]	2.6 ± 0.0[f]
		14.5				6.37 ± 0.85[cd]	2.2 ± 0.0[b]

Values represent mean ± S.E. means followed by the same letter are not significantly different at the level of confidence as per DMRT test (p≤0.05).

relationship between the increase in TDZ concentration up to the optimal level (2.83 µM) and the number of shoots per explants (Table 1). A maximum (16.48 ± 0.72) number of shoots developed within 8 weeks of culture (Figure 1a). Induced shoots at this stage attained a length of 2.60 ± 0.14 cm. Results obtained are in agreement with those of *Stevia rebaudiana* Bert (Lata et al., 2013). The maximum (24.57±0.37) number of shoots were obtained on BM supplemented with TDZ (2.83 µM) and NAA (0.1 µM) after 8 weeks of culture (Figure 1b). However, on IAA amended medium fewer shoots were produced as compared to IBA and NAA. The synergistic effect of TDZ in combination with other auxin has been demonstrated in the case of *Stemona hutanguriana* (Prathanturarug et al., 2012). By repeated sub-culturing on MS with TDZ (2.83 µM) and NAA (0.1 µM) number of shoots increased (45±1.33) after five times of sub cultures (each of 21 days) beyond which there is gradual decline in multiplication rate. This enhanced rate of shoot multiplication by subsequent subcultures substantiates with the earlier reports on *Ipomoea batatas* L (Sefasi et al., 2013) and *Andrographis neesiana* (Karuppusamy et al., 2010). Well developed microshoots of *P. lanceolata* were transferred to half strength MS medium containing IAA, IBA and NAA. The highest rooting frequency (100%) with maximum of (7.67±0.44) roots per shoot were achieved with 0.1 µM NAA (Figure 1c) where profuse rooting was observed. Similar results have also been achieved in *Arnebia hispidissima* (Shekhawat and Shekhawat, 2011) and regenerated plantlets were transferred to thermacoal cups consisted of sterilized garden soil and compost (1:1) as potting medium and maintained under controlled condition for two months prior to transfer to earthen pots containing garden soil the regenerated plant showed homogeneity with no phenotypic changes (Figure 1d). To confirm the genetic fidelity of the regenerated plants maintained in culture period of 6 months, RAPD analysis was carried out. The

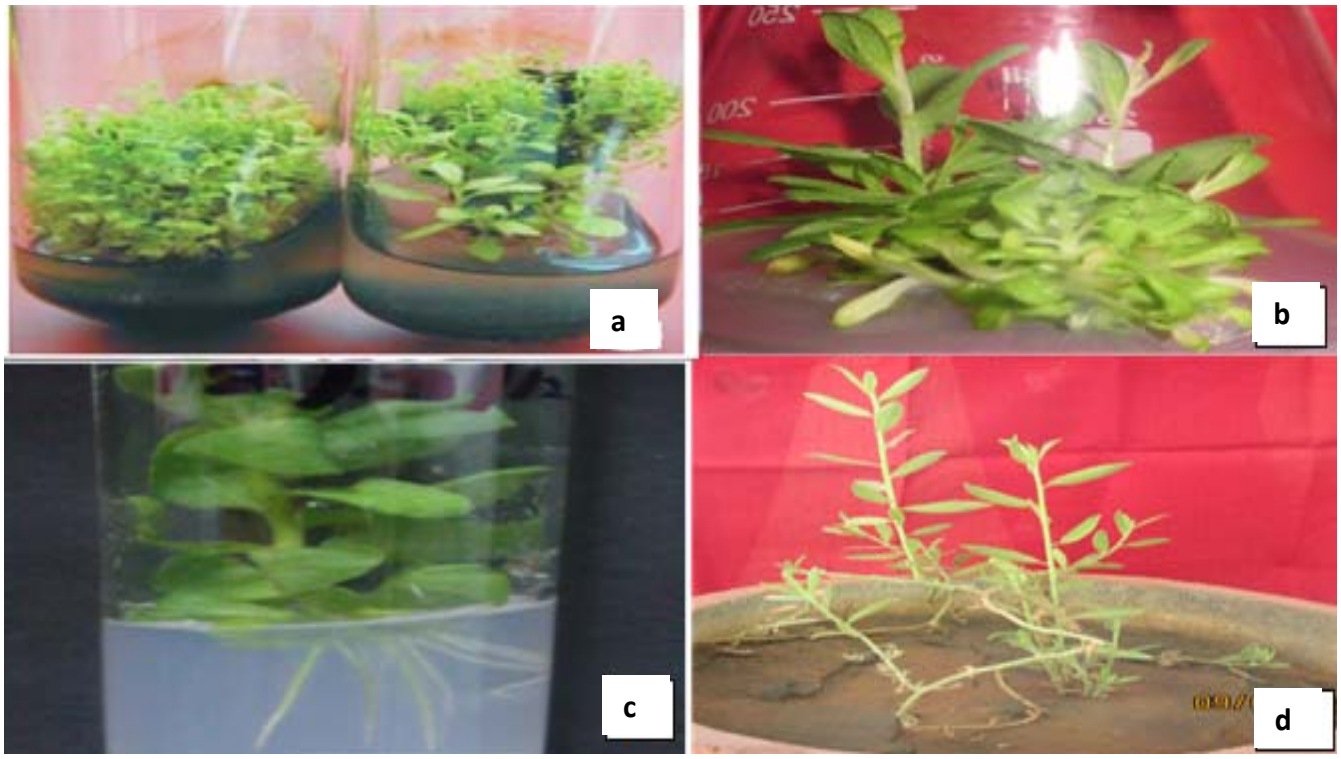

Figure 1. *In vitro* regeneration of *P. lanceolata*. **(a)** Multiple shoot induction from nodal segments cultured on BM + TDZ (2.83 µM); **(b)** Enhanced shoot multiplication and elongation on BM + TDZ (2.83 µM) + NAA (0.1 µM); **(c)** Rooted shoot plantlets on 1/2 strength MS with NAA (0.1 µM). **(d)** Acclimatized plants.

selected RAPD primers generated a total number of 87 bands, which were monomorphic for all the analyzed plants, including the control plant (Figure 2). Primer generated amplification products ranged in size from 226 to 1500 bp. A total of 87 bands were produced using 4 primers with 86 monomorphic and 1 polymorphic band (Table 2). Among treatments which were produced from different subcultures and mother plant, the range of similarity coefficients was from 0.93 to 1.00. Based on the dendrogram (Figure 3), 2 main clusters were obtained. Cluster 1 contained CC1, CC3, CC4, CC5 and CC6 which had similarity at the coefficient level of 1.00. Cluster 2 was occupied by CC2 corresponding to the first sub-culture which showed similarity at the coefficient level of 0.93 with cluster 1. The closest similarity with the mother plant was observed in the case of CC3, CC4, CC5 and CC6 which gave 100% similar RAPD profile to its mother plant. On the contrary, CC2 showed highest divergence with similarity value of 0.93 from MP (Table 3). The results are in contrast to inherited modifications in plants regenerated from tissue culture for *Dieffenbachia* cv. Camouflage (Shen et al., 2007), and in banana cultivar Valery, (Sheidai et al., 2008) and similar to *Tylophora indica* (Haque and Ghosh, 2013) where the genetic variation was induced with the time-period of the sub-culture. In the present study, it was found that minimal or no changes occurred between the MP and the sub culture produced and the variation which emerged after first sub culture reverted to the normal in the subsequent sub culturing.

Arya and Patni (2007) conducted a study on *P. lanceolata* using BAP (0.25 mgl^{-1}) and Kn (0.5 mgl^{-1}) for shoot induction on MS medium and obtained maximum number of shoots (30), while the present investigation give rise to quick and possibly less expensive method with more number of shoots at lower concentration of sucrose (2%) and lower concentration of growth regulator TDZ (2.83 µM) and the auxin used for rooting was at lower concentration NAA 0.1 µM and the rate of survival was 100% as compared to 70% from the previous study.

Conclusion

The study shows that plants regenerated through nodal culture *in vitro* could be successfully used for clonal propagation with very little risk of somaclonal variation and RAPD could be a good molecular marker to evaluate the genetic stability, to regenerates the *ex situ* conservation of this important medicinal plant.

Conflict of Interests

The author(s) have not declared any conflict of interests.

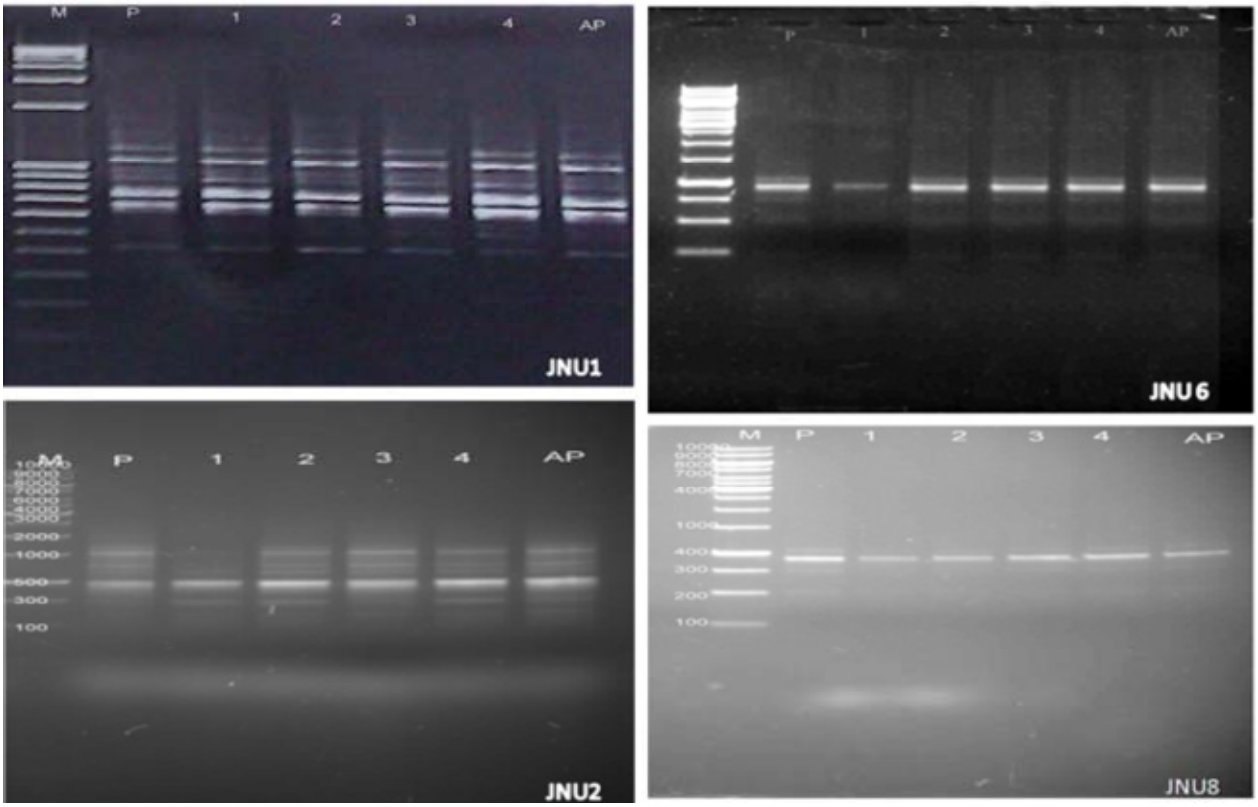

Figure 2. RAPD banding profile in *P. lanceolata* showing bands with JNU1, JNU2, JNU6 and JNU8.

Table 2. Total number and size range of amplified product and number of polymorphic bands generated by 4 (14mers) primer in micropropagated and mother plants of *P. lanceolata*.

Primers	Sequence 5'-3'	Scorable bands	Polymorphic bands	Monomorphic bands	Size Range	% Monomorphism	% Polymorphism
JNU1	CAGCCGCGGATCGT	36	0	36	1350-420	100	0
JNU2	CCCCCGGACCCAAA	27	0	27	412-200	100	0
JNU6	TTTCGGGGCCTTGG	11	1	10	390-400	100	9.09
JNU8	AGGGGCACGGATGC	12	0	12	410-380	100	0

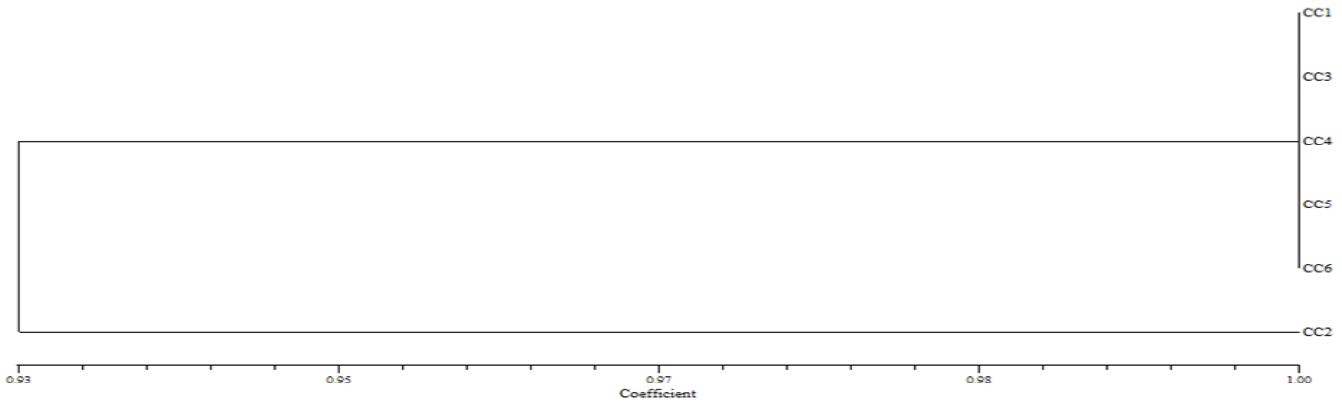

Figure 3. Dendogram of *P. lanceolata* showing genetic similarity among the *in vitro* generated plants and mother plant by UPGMA analysis based on single primers.

Table 3. Similarity matrix of the somaclonal variation between mother plant and different subcultures in *P. lanceolata*.

Cluster	CC1	CC2	CC3	CC4	CC5	CC6
CC1	1					
CC2	0.9333	1				
CC3	1	0.9333	1			
CC4	1	0.9333	1	1		
CC5	1	0.9333	1	1	1	
CC6	1	0.9333	1	1	1	1

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Conflict of Interests

There is no conflict of interest among the authors.

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