Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-021-02108-0

RESEARCH NOTE

Somatic embryogenesis as a tool for reproduction of genetically stable plants in banana and confirmatory field trials

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Received: 11 December 2020 / Accepted: 19 May 2021
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Abstract

Somatic embryogenesis is an important tool for crop improvement through transgenic approach and even for gene editing. It has been hypothesized regularly for large-scale propagation of banana which necessitates basic data on genetic fidelity and field performance of the plants towards ensure the commercial feasibility of the technique. Plantlets regenerated from embryogenic cell suspension (ECS) cultures established using immature male flower buds were examined for genetic fidelity using Inter Simple Sequence Repeats (ISSR) markers. Results showed that the primers UBC 808, UBC 811 and UBC 841 each generated one polymorphic band with an overall variation in banding pattern of 3.34 and 2.09% in cvs. Grand Naine and Rasthali respectively. Field evaluation of the ECS derived plants showed that there were no negative effects on the vegetative and yield parameters. Remarkably no phenotypic off-types were observed in this field trial. The level of genetic variation observed in this study is not an obstacle for further uptake of this novel propagation technique.

Key message

Field performance of ECS derived plants being on par with shoot tip cultured plants concludes that somatic embryogenesis could be successfully employed for commercial propagation of banana plantlets.

Communicated by Bart Panis.

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Published online: 10 June 2021

Keywords Genetic fidelity · Field performance · Agronomic traits · Tissue culture · Field evaluation

Introduction

Banana and plantains (Genus *Musa*, family *Musaceae*) are the most valued crop plants of the tropics and sub-tropics across the world and account for more than US$45 billion worth international trade with a huge impact on the economy of many countries (FAO 2020). In terms of volume, banana ranks second among fruit crops with an annual production of 115.7 million tonnes of bananas (75%) and 39.5 million tonnes of plantains (25%) from an area of 8.72 million hectares, led by India and China with a combined total of 28% of global production (FAO 2020). Cavendish group of bananas are the mainstay of banana industry, followed by a wide range of cultivated banana varieties like Silk (AAB), Plantains (AAB), Mysore (Poovan-AAB), Red banana (AAA), Pisang Awak (ABB), and cooking bananas (ABB). Grand Naine (AAA) has a huge demand in both local and international markets for its exclusive taste and texture. In Asia and more specifically in India, consumer preference for Rasthali (Silk group), has made it popular and highly priced for its fruit quality (Hazarika et al. 2014) and therefore has a potential niche both in the local and world markets as a premium dessert variety similar to cavendish bananas (Kheng et al. 2012). In India, 55% of the banana growing area is occupied by cavendish bananas and more specifically, Grand Naine, and cv. Rasthali, occupies one eighth of the area under non-cavendish bananas. Approximately 20% of Grand Naine and less than 1% of Rasthali plantations are by tissue culture and planting materials mainly derived through shoot tip culture.

The banana tissue culture industries mainly depend on shoot tip culture technique and spend almost a year for meristem multiplication and eventual rooting and hardening, before the planting season. Now the process is considered as expensive and looking for alternative techniques. Thus, the alternative mass propagation technique through somatic embryogenesis (SE) followed by embryogenic cell suspension (ECS) has been reported in recent past (Kumaravel et al. 2017, 2020a, c; Uma et al. 2019; Marimuthu et al. 2019). SE is a process for induction of somatic embryos from a vegetative tissue/explant later which germinate as whole plant like zygotic embryos. Escobedo-GraciaMedrano et al. (2016) reviewed SE in banana with the focus on different explants, media composition, ECS establishment, somaclonal variation and genetic transformation. Commercialization of this technique for mass propagation requires intensive studies on the basic process of transition from vegetative state to embryogenic state, genetic fidelity of the plantlets and field performance of planting material. SE is highly genome dependent as the efficiency varies with cultivars. Kumaravel et al. (2017, 2020a, c) has analyzed proteomics, antioxidant enzymes and endogenous hormones during developmental stages of SE and enhanced the efficiency of SE in banana. ECS establishment and multiplication using a bubble column balloon type bioreactor has been achieved in commercial cultivars of banana (Uma et al. 2019; Karthic et al. 2017a, b). But the success of any micropropagation technique is not only the higher regeneration efficiency, but its genetic fidelity and field stability are very vital. In banana, the genetic fidelity of regenerated plants is often questioned because of the occurrence of somaclonal variations in micropropagated plants (Martins et al. 2004). The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerants are reported to seriously limit the broader utility of the micropropagation system (Saraswathi et al. 2016, 2020; Salvi et al. 2001). Meager reports showed the genetic fidelity of somatic embryo derived plants using different molecular markers (Morais-Lino et al. 2016; Nandhakumar et al. 2018; Natarajan et al. 2020). However, somaclonal variation is a major constraint in every new protocol for large scale propagation of banana.

Materials and methods

Sword suckers and male flower buds from healthy banana plants of cvs. Grand Naine (AAA) and Rasthali (AAB) were used as explants. In vitro shoot tip culture was carried out as per the protocol described by Saraswathi et al. (2016). ECS of banana cvs. Grand Naine and Rasthali were established as per the protocol described by (Kumaravel et al. 2017, 2020b). Immature male floral hands of both cultivars turned into callus within 3–8 months of initiation in callus induction medium (MA1 semi-solid medium supplemented with 4 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D), 1 mg/L Indole 3 acetic acid (IAA),1 mg/L 1-naphthaleneacetic acid (NAA) and 30 g/L sucrose). The embryogenic calli (EC) were formed from 8 to 16th position of floral hands. Friable EC were transferred into liquid suspension medium (20 mL of M2 liquid medium supplemented with 1 mg/L IAA, 1.1 mg/ L 2,4-D, 250 µg/L zeatin (ZEA; Sigma-Aldrich®, St. Louis, MO) and with a pH adjusted to 5.8 for multiplication of embryogenic cells (Strosse et al. 2003). Homogenous ECS was obtained within 10–15 subcultures of 10 days intervals. ECS (one ml) was inoculated in M3 semisolid medium supplemented with 200 µg/L NAA, 80 µg/L kinetin (Sigma-Aldrich®), and 40 µg/L ZEA with a final pH of 5.8 (Strosse et al. 2003). Within 60–90 days ECS completely matured as somatic embryos in both the cultivars. The embryos germinated into plantlets on semisolid germination medium

supplemented with 0.5 mg/L 6-benzylaminopurine (BAP; Sigma-Aldrich®) and 2 mg/L IAA with a final pH of 5.8 (Strosse et al. 2003). The germinated plantlets were further transferred to rooting medium and hardening (Fig. 1). Leaf samples from fifteen plantlets obtained through ECS were selected randomly in the secondary hardening stage and used for genetic fidelity analysis. Sucker and shoot tip derived plants of test cultivars were used as controls. Genomic DNA was isolated using CTAB method as stated by Gawel and Jarret (1991) with minor modifications. The purity of genomic DNA was checked on a 0.8% agarose gel and the concentration of DNA was determined by a UV–visible spectrophotometer from Perkin Elmer (UV–vis Lambda 25, USA). Around eight pairs of ISSR markers were used for the genetic fidelity test. The primers used for the analysis are listed in the Table 1. The PCR was carried out on gradient master cycler (ProFlexTM Base, Applied Biosystems, Singapore) in 25 µL reaction containing 15 ng of genomic DNA, 100 µM of dNTPs, 0.2 µM of primers, 1X PCR buffer and 1 U of Taq DNA polymerase enzyme. The PCR-amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels (BIO-RAD Laboratories Inc., Hercules, CA, USA) at 100 V for 3–4 h. The PCR-amplified ISSR marker products were scored across the different source samples and compared with the controls (Table 1). Suckers (3–5 kg) and in vitro raised and hardened plants (ECS and shoot tip culture derived) had attained a height of 40–50 cm, girth of 5–10 cm with 5–6 photosynthetically active leaves, and were transferred to the field. A total 300 plants of cv. Grand Naine (each source 100 plants) were planted in commercial plantations located at Kamagoundanpatti, Theni, Tamilnadu (10°03′57.5″ N 77°30′30.0″ E). Similarly, 300 plants of cv. Rasthali (each source 100 plants) were planted and maintained in the research farm located at Pothavur village, Trichy (10°51′28.6″ N 78°46′50.1″ E). Pits were established for 1 × 1 × 1 (height × length × breadth) foot size. The suckers and in vitro raised plants were planted in the pit along with 5 kg of farmyard manure and 150 g

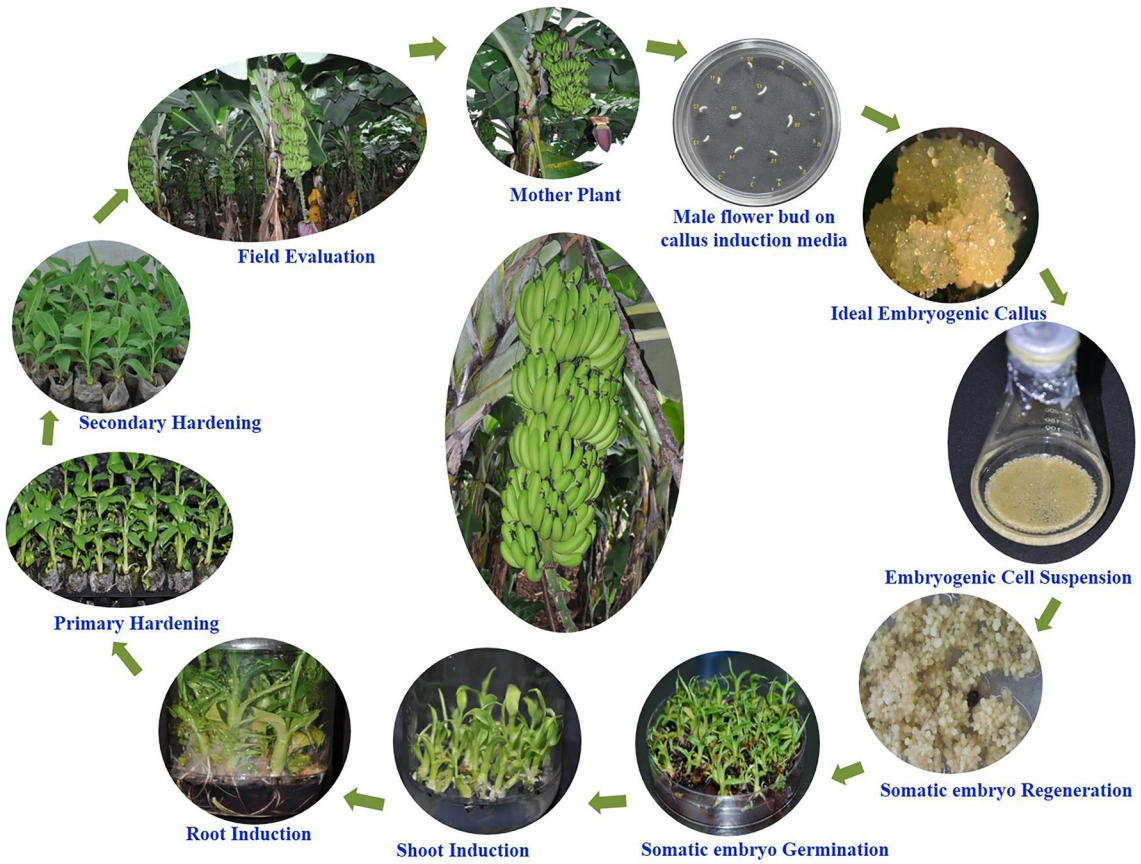

Fig. 1 Schematic representation of plantlets regeneration through somatic embryogenesis in banana. Cultures of callus induction to root induction were maintained in maintained at 24 ± 2 °C with relative humidity (RH) with 50–60% and cultures were maintained in dark condition up to the stage of somatic embryo regeneration

Table 1 List of ISSR primers used for assessment of genetic fidelity and DNA amplification profile of ECS derived plants of banana cultivars Grand Naine and Rasthali

Primer	Sequence	Annealing temperature	No. of bands (Monomorphic-polymorphic-total)	
			cv. Grand Naine	cv. Rasthali
UBC 807	AGAGAGAGAGAGAGAGT	46.8	12–0–12	14–0–14
UBC 808	AGAGAGAGAGAGAGAGC	50.6	10–1–11	10–0–10
UBC 810	GAGAGAGAGAGAGAGAT	50.4	10–0–10	08–0–08
UBC 811	GAGAGAGAGAGAGAGAC	46.0	09–1–10	12–1–13
UBC 834	AGAGAGAGAGAGAGAGYT	54.0	12–0–12	12–0–12
UBC 836	AGAGAGAGAGAGAGAGYA	51.0	08–0–08	10–0–10
UBC 840	GAGAGAGAGAGAGAGAYT	54.0	10–0–10	09–0–09
UBC 841	GAGAGAGAGAGAGAGAYC	46.6	12–1–13	10–1–11
Monomorphic band (%)			96.65	97.9
Polymorphic band (%)			3.34	2.09

of Superphosphate as basal nutrients. In addition, as a soil nematode controlling agent 30 to 50 g of CALDAN (Cartap Hydrochloride) granules were added in the pits to manage nematodes. Fertilizer dosages were given to the plants during third month (150 g Urea + 200 g Monophosphate + 200 g Superphosphate/plant), fifth month (150 g Urea + 250 g Monophosphate/plant) and seventh month (200 g Urea + 200 g Monophosphate/plant). Data on pseudostem height and girth (cm), number of leaves at shooting, bunch weight (kg), number of hands per bunch, and total duration of crop (days) were recorded.

Results and discussion

There were 86 and 87 scorable bands from eight primers ranging from 200 to 1500 bp in cvs. Grand Naine and Rasthali respectively (Fig. 2). Three primers (UBC 808, 811 and 841), each generated one polymorphic band with overall variation in banding pattern of 3.34 and 2.09% in cvs. Grand Naine and Rasthali respectively. This phenomenon of variation in the intensity of some fragments has also been observed in banana by Aremu et al. (2013) and Bairu et al. (2006) based on the concentration of BAP used and passage of subcultures maintained. However, Saraswathi et al. (2016) developed a cost-effective in vitro shoot tip culture protocol for large-scale propagation of banana and substantiated with below 15% of polymorphism. Sahijram et al. (2003) reported that, up to 10% variation is permitted mainly because of its flexibility of genetic make-up (Cote et al. 1993). Dhed'a et al. (1991) reported that the plantlets production from ECS has high level of genetic stability. Since, embryogenic cells under appropriate conditions are able to develop into a complete functional embryos due to the cellular totipotency and produce fewer rates of somaclonal variations (Smitha and Nair 2020). Plantlets regenerated from ECS did not show any genetic variation by SSR and ISSR marker analysis (Nandhakumar et al. 2018; Morais-Lino et al. 2016). This minimal variation observed in this study could be hypothesized as follows. (i) The prolonged maintenance of ECS in 2, 4-D supplemented medium may cause asynchronous cellular and physiological processes may lead to chromosomal instability. (ii) The process of conversion of cells into somatic embryos in a semisolid substrate for prolonged duration may cause insufficient supply of nutrients to the cells and simultaneous oxidative stress-inducing compounds increase the cell endogenous auxin levels may induce SE and DNA methylation. Sharma et al. (2007) reported that the DNA methylation is an indispensable process in SE, although it is essential to control within certain limits. Nevertheless, Dhed'a et al. (1991) reported that the 5–10% of abnormal embryos observed in SE of banana cv. Bluggoe, the plants of which turned out to be normal after field establishment resulting in minimum somaclonal variants. The level of variation observed in this study is not an obstacle for further uptake of this novel propagation technique. But it is essential to validate the SE derived plants under field condition.

The results of field data is presented in Table 2 showed no negative effects on the vegetative and yield parameters of the plants regenerated from ECS remarkably no phenotypic off-types observed in this field trial (Fig. 3). In vitro raised plants showed improved vegetative and yield characters than sucker plants of both the cultivars even in ratoon crop. There was a significant difference in total crop duration in first and ratoon crop. The total crop duration in sucker plants was higher than in vitro derived plants. Earlier studies reported that the growth and yield performance of in- vitro propagated plants were superior than the sucker plants of banana and plantains (Nandhakumar et al. 2018; Buah et al. 2000; Cote et al. 2000). Our results correlated with these

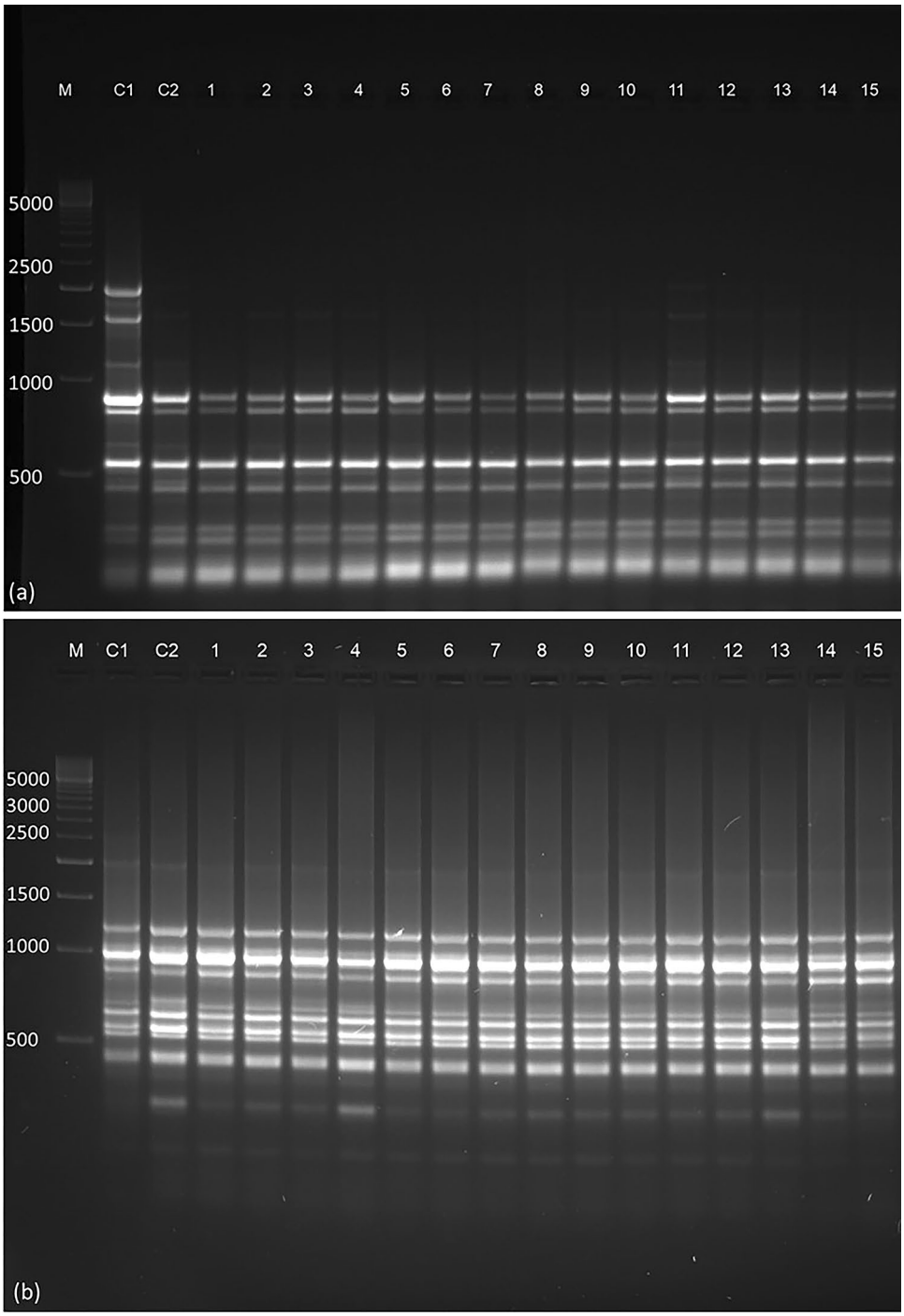

Fig. 2 ISSR profiles of the sucker plant, shoot tip culture derived plantlets and ECS derived plants of banana. **a** DNA banding pattern of Grand Naine (UBC 808), **b** DNA banding pattern of Rasthali (UBC 807). Lane M: DNA Marker ladder. Lane C1: DNA banding pattern of the sucker plant. Lane C2: DNA banding pattern of acclimated plants that were raised from shoot tip culture. Lane 1–15: DNA banding pattern of acclimated plants that were raised from ECS

results. Higher vigor of in vitro raised plants was explained in earlier studies that the plants possess an active root and shoot system and also had physiologically active leaves that start functioning instantly after planting in the field. On the other hand, sucker plants have to develop fresh root and leaves from rhizome and then start photosynthesis after 5 to 6 weeks of planting (Robinson et al. 1993; Drew and Smith 1990). Another important factor for the delayed growth of sucker plants is physical damage caused to the roots while separating from the mother plant. Hence, it has been concluded that the ECS derived planting material of banana cvs. Grand Naine and Rasthali performed well in this field trial and this novel propagation technique could be utilized for large- scale propagation with a need for defining a good laboratory practice. Furthermore, somatic embryogenesis in an automated system and a large-scale field evaluation may restrict further dillydally on commercial use of the technique.

Table 2 Evaluation of vegetative traits and yield potential of different source of planting material in banana cultivars Grand Naine (AAA) and Rasthali (AAB)

Source for planting material	Pseudostem height (cm)		Pseudostem grith (cm)		No. of leaves at shooting		Bunch weight (kg)		No. of hands per bunch		Total crop duration (days)	
	Grand Naine	Rasthali	Grand Naine	Rasthali	Grand Naine	Rasthali	Grand Naine	Rasthali	Grand Naine	Rasthali	Grand Naine	Rasthali
First crop												
Sucker	223 ± 14^{a}	273 ± 15^{b}	75.4 ± 2.1^{b}	71.8 ± 2.5^{a}	11.2 ± 1.7^{b}	13.2 ± 1.4^{b}	28.3 ± 4.5^{ab}	12.7 ± 4.8^{a}	12.1 ± 2.5^{b}	7.2 ± 1.7^{ab}	360 ± 9.7^{a}	452 ± 7.5^{a}
Shoot tip	228 ± 9^{a}	290 ± 7^{a}	80.5 ± 0.5^{a}	73.5 ± 1.5^{a}	12.1 ± 0.7^{ab}	15.1 ± 0.3^{ab}	32.1 ± 2.4^{a}	13.6 ± 2.3^{a}	11.0 ± 1.1^{a}	8.0 ± 1.0^{a}	352 ± 2.2^{b}	435 ± 4.5^{b}
ECS	233 ± 7^{a}	289 ± 11^{a}	79.6 ± 1.0^{a}	72.5 ± 1.7^{a}	12.2 ± 1.4^{a}	15.6 ± 1.6^{a}	32.5 ± 2.7^{a}	13.8 ± 2.2^{a}	12.1 ± 0.3^{a}	8.0 ± 1.1^{a}	350 ± 3.9^{c}	433 ± 3.2^{b}
Ratoon crop												
Sucker	228 ± 7^{b}	278 ± 11^{b}	75.8 ± 2.2^{c}	72.5 ± 1.3^{a}	11.5 ± 1.5^{b}	14.3 ± 1.5^{a}	29.3 ± 5.1^{ab}	12.8 ± 2.4^{a}	12.3 ± 1.9^{a}	7.1 ± 1.4^{b}	355 ± 6.5^{a}	445 ± 6.7^{a}
Shoot tip	228 ± 5^{b}	291 ± 15^{a}	81.7 ± 0.5^{b}	73.8 ± 1.2^{a}	13.1 ± 0.7^{a}	15.1 ± 0.7^{a}	32.5 ± 3.3^{a}	13.8 ± 1.9^{a}	11.3 ± 0.7^{a}	9.0 ± 1.2^{a}	348 ± 7.2^{b}	432 ± 3.5^{b}
ECS	239 ± 5^{a}	291 ± 9^{a}	82.5 ± 0.3^{a}	73.3 ± 1.5^{a}	12.3 ± 0.7^{ab}	15.2 ± 1.5^{a}	34.1 ± 1.1^{a}	13.9 ± 2.1^{a}	12.4 ± 0.3^{a}	8.0 ± 1.3^{a}	345 ± 3.5^{b}	430 ± 2.4^{b}

Each value represents the treatment means of 300 independent replicates ± SD. Values with same letter with in columns are not significantly different according to DMRT at $p = 0.05$ level. Statistics was carried out within the group of first and ratoon crop

Fig. 3 Field evaluation of ECS derived banana plants. **a** and **b** cv. Rasthali in field, **c** and **d** cv. Grand Naine in field

Acknowledgements Authors thank the farmer Mr. Amayaraj for providing land to carry out the field trial at Theni district, Tamilnadu, India. Authors gratefully acknowledge the Director, ICAR- NRCB-Trichy for providing the laboratory facilities to carry out the research.

Author contributions SU, MK, and SB designed the experiment. SU, MK, MSS and SB collected plant samples from *in- vitro* culture condition. SU, MK, PD and RK collected field data. All authors had written the manuscript. SU, SB and MSS monitored the Research. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors have no conflict of interest.

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