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Full Length Research Paper

Callus induction of *Tacca integrifolia* Ker Gawl using stem nodal segment

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A study was conducted to determine the optimum concentration of plant growth regulators on *in vitro* callus induction of *Tacca integrifolia* using stem nodal segment. Fresh, dry weight and morphology of callus were evaluated and the results showed significant effects on callus induction when analyzed at a 5% level of significance. Among the treatments, Murashige and Skoog with only 6-Benzylaminopurine (1.0 mg/L) produced the highest result of fresh weight (1.2637 ± 0.14 g) and dry weight (0.1204 ± 0.01 g) and appeared the compact and green calli. Besides, the lowest results were 2,4-dichlorophenoxyacetic acid (2.5 mg/L) which only produced the fresh weight (0.2812 ± 0.04 g) and dry weight (0.0271 ± 0.00 g) and appeared friable and yellowish. The result of this study has revealed that the presence of 2,4-Dichlorophenoxyacetic acid or 1-Naphthaleneacetic acid in combination with 6-Benzylaminopurine does not giving much impact on callus induction based on mass production in addition, while 6-Benzylaminopurine alone, can produce more calli.

Key words: White bat flower, compact callus, Dioscoreaceae, 6-Benzylaminopurine (BAP).

INTRODUCTION

Tacca integrifolia, a white bat flower, is one of the tropical herbs belonging to the *Tacca* genus of the Taccaceae family, widely distributed in Southeast Asia (Zhang et al., 2006). It was classified under flowering plants in the yam family Dioscoreaceae after molecular research (Caddick et al., 2002). Nonetheless, both families still share a close taxonomic relationship (Borokini and Abiodun, 2012). *T. integrifolia* has several local names as 'Belimbing Tanah', 'Keladi Murai' and 'Janggut Adam' and this species very closely resembles *Tacca chantrieri* but differs only in petiolar-like sheath in the bract (Misrol et al., 2015; Baruah et al., 2015). The potential traditional medicine for this species was being practised by the Thai and Myanmar folk people to fight against the pest, control blood pressure, improve sexual function in humans, treatment for skin diseases and various kinds of cancers (Misrol et al., 2015; Hossen et al., 2016; Shwe et al., 2010).

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Tacca cristata Jack which is synonymized with *T. integrifolia* Ker Gawl due to their similar inflorescences (heteromorphic bracts: spathulate inner bracts and ovate outer bracts) believed to have antidiabetic properties in Malay folk medicine besides treatment of hypertension, haemorrhoids and heart failure (Nurraihana et al., 2016). Moreover, when the extracts from the tuber and roots were mixed together with *Goniothalamus malayanus* (Mempisang), it makes a good treatment for the kidney (Jamaludin and Mohamad, 2016). However, based on Jiang et al. (2014), *T. integrifolia* is mutagenic and its combined extracts from the medicinal plants are highly cytotoxic to the human cell lines, Hep2 and HFL1. In recent studies, Taccaceae are believed to produce specific secondary metabolites that have the potential for anti-cancer because they contain taccalonolide (Hapsari et al., 2018).

Seeing that this herbaceous plant is only grown explicitly in the wild forest and generally used in Southeast Asian countries for medicinal purposes, there is inadequate information on how to propagate this plant and bat flowers can be propagated from seeds and stem budding yet plants and seeds are rarely collected from the natural forest (Abd Razak et al., 2007). Nevertheless, some studies on *Tacca in-vitro* are limited; however, these initial studies confirmed the potential of Tacca plants' response to *in vitro* cultures and provide profitable data for further research. Hence, much more attention should be emphasized to *Tacca* species for further phytochemical, pharmacological and cultural studies. For the conservation aspect, overexploitation of the bioactive constituents can be minimized by practicing the *in vitro* techniques.

For all that, this research is an experiment to determine the optimum concentration of auxin and cytokinin on *in vitro* callus for *T. integrifolia*. Since *in vitro* callus is an alternative source to produce secondary metabolite production and generated callus culture could be utilized as an alternative and easy way for better screening, isolation and identification of secondary metabolites. In order to evaluate callus growth, the callus texture can act as one of the markers whereas the friable texture indicates the development of the somatic embryo and the solid compact texture also difficult to be released, will indicate growth into organogenesis.

MATERIALS AND METHODS

Explant selection

In the present experiment, the plantlet was grown in *in vitro* conditions and was maintained with periodic subculturing at an interval of four weeks at 25 ± 2°C, under a 16 h photoperiod. The sterilized stem nodal segments obtained from *in vitro* grown plants were cut approximately 0.5 to 1.0 cm in length and immediately inoculated aseptically in the Petri dish on the media supplemented with different sets concentrations and combinations of plant growth regulators.

Medium composition

The basal medium used for callus induction consisted of MS mineral salts with vitamin, 3% (W/V) sucrose, and 0.8% agar. The pH of the media was adjusted to 5.7 to 5.8. The plant growth regulators such as BAP, 2,4-D, NAA and Kinetin were added to the media before autoclaving for 20 min at 121°C and after being autoclaved, the medium was poured into several vials with an appropriate amount.

Effect of plant growth regulators on callus induction

Four hormones were involved in this experiment: 1-Naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin, respectively. Different concentrations for each hormone are stated: NAA (0.1-2.0 mg/L), BAP (0.1-1.5 mg/L), 2,4-D (0.5-2.5 mg/L), BAP (0.1- 1.5 mg/L) with a constant concentration of 1.0 mg/L NAA, 2,4-D (0.5-2.5 mg/L) with a constant concentration of 1.5 mg/L Kn, BAP (0.1-1.5 mg/L) with a constant concentration of 0.5 mg/L 2,4-D and control (MS). The cultures were kept in dark conditions during the first month of culture before being kept under light (2000 lux) with a photoperiod of 16 h light at 25 ± 2°C. Callus induction was observed after 8 weeks when the fresh and dry weight was taken. The dry weight of the callus was obtained after drying to a constant weight at 45°C for 24 h. Besides, the morphology observation was recorded as the callus colour and texture. All experiments were repeated twice using 5 replicates (Petri dish) each containing three explants.

Statistical analysis

All the experiments were conducted using Randomized Completely Block Design (RCBD) which uses at least five replicates for each treatment. All the data was analyzed with one-way analysis of variance (ANOVA) using the SPSS V21 statistical program and the mean was compared by using Duncan's multiple range test at P=0.05. In all cases, a p-value of 0.05 or less was considered statistically significant.

RESULTS AND DISCUSSION

Callus induction for *T. integrifolia* was studied by using seven treatments of plant growth regulator (PGRs) including control (MS) media. Leaf as an explant to initiate the callus induction was reported as the best by other researchers (Khajuria et al., 2017; Pawar et al., 2018; Mahmuda et al., 2019) including a study on *T. chantrieri*. However, there is a lack of evidence showing that leaf is the best explant to induce the callus in Dioscoreaceae family. Hence, stem nodal segments were used as an explant source to induce the callus in the Petri dish. All the explants containing treatment except control successfully produced callus with the variable response (Table 1). The interactions among the different concentration for all treatments showed a significant *p*-value ≥ 0.05 which the null hypothesis (H_0) were rejected. There were significant differences in fresh and dry weight results.

In the present study, the callus induction began within one month in a dark condition before being transferred

Table 1. Mean of fresh weight, dry weight, and morphology observation on callus induction of *Tacca integrifolia*.

Treatment	Fresh Weight (g) (Mean ± SE)	Dry Weight (g) (Mean ± SE)	Morphology observation
Control (MS)	-	-	No callus
MS + BAP			
0.1	0.3728 ± 0.01^{b}	0.0391 ± 0.00^{b}	Green, friable
0.5	0.4434 ± 0.05^{b}	0.0412 ± 0.00^{b}	Green, friable shooty callus
1.0	1.2637 ± 0.14^{a}	0.1204 ± 0.01^{a}	Green, compact
1.5	0.3963 ± 0.03^{b}	0.0406 ± 0.00^{b}	Green, compact
MS + NAA			
0.1	0.6126 ± 0.03^{b}	0.0636 ± 0.00^{b}	Green, friable
1.0	0.8622 ± 0.04^{a}	0.0860 ± 0.00^{a}	Yellowish, friable shooty callus
1.5	0.5951 ± 0.05^{b}	0.0585 ± 0.00^{b}	Yellowish, friable shooty callus
2.0	0.3748 ± 0.05^{c}	0.0349 ± 0.00^{c}	Yellowish, friable
MS + 2,4-D			
0.5	0.5295 ± 0.10^{a}	0.0733 ± 0.01^{a}	Green, friable shooty callus
1.0	0.3052± 0.03^{b}	0.0303 ± 0.00^{b}	Yellowish, friable shooty callus
2.0	0.3562 ± 0.04^{ab}	0.0362 ± 0.00^{b}	Pale green , friable
2.5	0.2812 ± 0.04^{b}	0.0271 ± 0.00^{b}	White, friable
MS + BAP + NAA			
0.1 + 1.0	0.5102 ± 0.05^{b}	0.0458 ± 0.00^{b}	Green, friable, shooty callus
0.5 + 1.0	0.8210 ± 0.05^{a}	0.0837 ± 0.01^{a}	Yellowish, friable
1.0 + 1.0	0.7999 ± 0.05^{a}	0.0765 ± 0.01^{a}	Yellowish, friable
1.5 + 1.0	0.6332 ± 0.07^{b}	0.0689 ± 0.01^{a}	Yellowish, friable
MS+ BAP+2,4-D			
0.1 + 0.5	0.3211 ± 0.03^{b}	0.0328 ± 0.00^{b}	Green, friable
0.5 + 0.5	0.4324 ± 0.03^{b}	0.0451 ± 0.00^{b}	Green, friable
1.0 + 0.5	0.8503 ± 0.11^{a}	0.0779 ± 0.01^{a}	Green, friable
1.5 + 0.5	0.6853 ± 0.05^{a}	0.0682 ± 0.01^{a}	White, compact
MS+ 2,4-D + Kn			
0.5 + 1.5	0.2981 ± 0.03^{c}	0.0300 ± 0.00^{b}	Pale Green, friable, shooty callus
1.0 + 1.5	0.7574 ± 0.10^{a}	0.0708 ± 0.01^{a}	Yellowish, friable
2.0 + 1.5	0.5847 ± 0.03^{b}	0.0585 ± 0.00^{a}	Yellowish, friable
2.5 + 1.5	0.4464 ± 0.03^{bc}	0.0425 ± 0.00^{b}	Yellowish, friable

MS= Murashige and Skoog; FW=fresh weight, DW=dry weight, SE=standard error.
Source: Authors.

into a light condition. From all the single hormones used as treatment contained with Murashige and Skoog (MS), BAP produced a higher amount of callus at all concentrations between 0.1-1.5 mg/L. The highest fresh and dry weight that was produced at the concentration of 1.0 mg/L BAP is 1.2637 ± 0.14 and 0.1204 ± 0.01 g, respectively (Table 1). Besides, the morphology that appeared on the callus is compact and green in colour (Figure 1). A study on callus induction in the herbaceous plant, patchouli by Mayerni et al. (2020) reported that the callus successfully formed without any combination and produced best at a concentration of 1.0 mg/L BAP. In line with the results of their finding, it showed that the best range of BAP concentrations that can be used to form callus from leaf or node explants is between 1.0 and 2.0 mg/L BAP. It differs from several studies that had been reported on callus response of the same plant *Tacca* genus which is *T. chantrieri,* the very close species

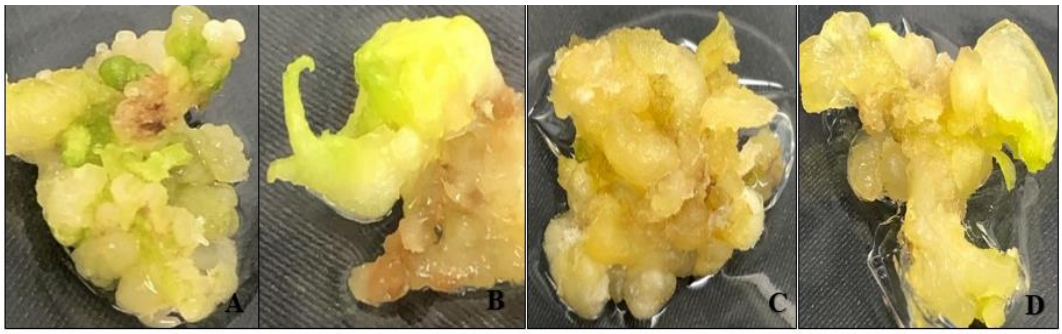

Figure 1. Callus induction from stem nodal segment explant on optimum treatment after 8 weeks of culture at 25 ± 2°C under light condition. a) 1.0 mg/L BAP. The calli formed greenish, compact and covered all the entire explants. b) The green and friable shooty callus appeared at 0.5 mg/L 2,4-D. c) The yellowish and friable callus appeared at MS+ 1.0 mg/L BAP + 0.5 mg/L 2,4-D. d) The pale green with friable shooty callus appeared at MS + 0.5 mg/L 2,4-D + 1.5 Kn.
Source: Authors.

related (Wei Ying et al., 2013; Yun Ping et al., 2012). However, in their research, they used leaf stalk and filament of flower explants with a trio combination MS + 2.0 mg/L BAP + 0.1 mg/L NAA + 0.1 mg/L 2,4-D. They found the best range of BAP concentration at 2.0 to 3.0 mg/L. Different responses might occur regarding the genotype variation and source of explants used for each species. Several researchers have found that the negative impact on callus formation occurred as increasing BAP to 2 mg/L while decreasing to 0.1 mg/L can reduce the rate of callus formation (Blinkov et al., 2022).

The following treatment, NAA with increasing concentration at 2.0 mg/L gave a lower amount of callus. Matsuoka and Hinata (1979) mentioned that when NAA at high concentration caused toxic, depressed callus growth also stimulated embryoid formation. This is proven by this study when the concentration increased; the value of fresh and dry weight gave a declined result. On the contrary, it differs from 1.0 mg/L NAA which is found as the best concentration and can produce more amount of callus weight with a compact callus on *Dioscorea rotundata* (Ezeibekwe et al., 2009). Nonetheless, the callus appears green and friable at low concentrations compared to the high concentration of NAA. Liu et al. (2018) have mentioned that the higher concentrations of NAA led to the formation of foamy, loose, and soft texture in calli. The green colour of the callus appeared due to the presence of light which promotes the synthesis of chlorophyll. Moreover, the decreased concentration of 2,4-D at 0.5 mg/L gave a higher amount of callus weight with green and friable shooty callus was observed. 2,4-D concentrations used between 0.5 and 2.5 mg/L were reported as the best treatments (Khajuria et al., 2017).

Since the callogenic response varies from hormonal concentration, they found that the lower concentration of 2,4-D alone or in combination with cytokinin is very promising for callus induction. Furthermore, the best media combinations that successfully produced the highest fresh and dry weight of callus were MS + 1.0 mg/L BAP + 0.5 mg/L 2,4-D with 0.9472 ± 0.17 g and 0.0789 ± 0.01 g, respectively while MS + 0.5 mg/L 2,4-D + 1.5 mg/L Kn gave the lowest weight of callus (Table 1). While in combination with Kinetin, 1.0 mg/L 2,4-D gave a higher weight of callus rather than 0.5 mg/L 2,4-D which only produced 0.2476±0.07 g fresh weight and 0.0326±0.01 g dry weight. This evidence is in line with the species study of *Barringtonia racemosa* (Nurul et al., 2013). BAP at a concentration between 0.5 and 1.5 mg/L is more suitable to be used when in combination with other PGRs such as NAA and 2,4-D. It was shown in this experiment that between that concentration, the callus weight produced higher when in combination with 0.5 mg/L 2,4-D or 1.0 mg/L NAA.

Conclusion

From the present study, it is revealed that the single hormone BAP at the concentration of 1.0 mg/L produce the highest amount of callus with 1.2637 ± 0.14 g fresh weight and 0.1204 ± 0.01 g dry weight with the morphology that appeared on callus being compact and green in colour. While MS + 0.5 mg/L 2,4-D + 1.5 mg/L Kn gave the lowest weight of callus which only produced 0.2476±0.07 g fresh weight and 0.0326±0.01 g dry weight. Plus, the callus appeared pale green in colour and friable shooty. In addition, the presence of 2,4-D or NAA in combination with BAP does not have much impact on the callus response. Further studies could be conducted since this finding was a starting point for enhancing the production of secondary metabolites. Besides, identifying novel approaches to utilize this plant in *in vitro* callus cultures for future use in pharmaceutical applications such as inducing the callus by using different

types of explant parts, different strengths of MS and other components.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation and molecular identification of tannase producing fungi from soil

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The study was conducted to isolate and identify tannase producing fungi from soil. Soil samples were collected aseptically and cultured on tannic acid agar (TAA) to screen for tannic acid hydrolysis. The observed isolates were sub-cultured to obtain pure culture and were later subjected to secondary screening in the Czapek medium. The biomass weight, tannase activity, and gallic acid concentration in the fermentation broth were determined using standard protocols. Fungal isolates with promising tannase activity were further used to produce tannase using acacia nuts and pine apple peel as sources of tannin. From the results obtained, a total of seven isolates showing good tannin degradation in both primary and secondary screening were identified by phylogenetic characterization as *Penicillium citrinum*, *Aspergillus niger* strain, *P. citrinum* isolate K9, *A. niger* strain 7806F, *Fusarium equiseti* isolate GS-WW-F14-13, *Aspergillus versicolor* isolate 777 and *A. niger* strain SCSGAF0145. Under submerged fermentation, *A. versicolor* had the highest tannase activity of 22.49±1.17 U/mL using acacia nuts as substrate. In conclusion, the isolated fungal strain is a good tannase producer which can be explored for industrial processes.

Key words: Fungi, tannase, gallic acid.

INTRODUCTION

Fungi have been exploited as a tool for manufacturing various highly important products, such as food in the form of single cell proteins, beverages, organic acids, and drugs. Bon et al. (2008) report that after antibiotics, enzymes are the main products obtained from fungi by biotechnological industries. Filamentous fungi play important role in enzyme production, because of their rapid growth rate on many substrates, ease of gene manipulation cum handling, and their ability to produce numerous biotechnological applicable metabolites. Of industrial importance is the enzyme tannase which has been reported to be secreted by various fungal strains (Aguilar-Zárate et al., 2014), such as *Aspergillus* which is considered to be the best producer, followed by

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Penicillium (Sabu et al., 2005). Other fungal strains reported in the literature to be tannase producers are *Fusarium* and *Trichoderma* (Raghuwanshi et al., 2011).

Tannase is a hydrolytic enzyme that breaks down tannin especially tannic acid into glucose and gallic acid. Industrially, tannase is used in the clarification of fruit juice that is rich in tannins and aimed at reducing the astringency of such products (de Lima et al., 2014; Selwal and Selwal, 2012). Gallic acid released from tannin degradation is used in the production of antibiotics, trimethoprim, used in the pharmaceutical industry, and pyrogallol or ester gallates which are used as food preservatives (Malgireddy et al., 2015). Tannase is also important in controlling environmental pollution by removing tannins, mainly polyphones present in tannery effluents.

Tannins are plant secondary metabolites described as the fourth most abundant group overhauled by polysaccharides: cellulose, hemicellulose, and lignin. Apart from their astringent nature which has been explored in drug development, nutritionally they are undesirable because they possess the ability to precipitate proteins from food (Girdhari and Peshwe, 2015; Murugan and Saleh, 2010). Tannins are readily available in agro-industrial waste and enhance their resistance to microbial attack. They are known to inhibit the growth of some microorganisms which slow down the rate of biodegradation to soil organic matter, thus, constituting an environmental pollutant. Consequently, tannase producing microorganisms can use such agro-industrial waste as a substrate for secreting tannase which hydrolyses tannin to yield gallic acids in large quantities.

Waste materials such as cashew husk, rice bran, plantain flour, banana peel (Gaayathiri et al., 2020), almond and mango leaves (Ire and Nwanguma, 2020), wheat bran, eucalyptus leaves, pomegranate peel, banana peel, and guava (Ahmed et al., 2020) have been used as the source of tannins. The use of these materials helps to reduce environmental pollution and serves as the source of single cell protein and production of the enzyme tannase (Seth and Chand, 2000). The present investigation was aimed at isolating fungal tannase from soil samples and determining the ability of the isolated fungi to degrade tannin-rich agricultural wastes (groundnut husk and acacia nuts).

METHODOLOGY

Materials and chemicals

The investigation only utilized analytical-grade compounds.

Sample collection

Samples of five soil were collected from soil down different tannin-rich plants (Almond, Malay apple, Neem, Banana and Cassia) within the premises of Lagos State Polytechnic, Ikorodu, Lagos State, Nigeria and were transported to the laboratory for direct use.

Media

The main screening of fungal isolates and the isolation of tannase-producing fungi were done using tannic acid agar medium (TAA) (Pinto et al., 2001). The following ingredients were used in its preparation: 10 tannic acid; $NaNO_3$ 3; KCl 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; KH_2PO_4 1.0; $FeSO_4 \cdot 7H_2O$ 0.01; agar 30. The medium was sterilized for 15 min at 121°C and adjusted to pH 4.5 +/- 0.2. Tannic acid solution was added to the medium after being sterilized separately using a membrane filter with a 33 mm diameter and 0.22 m pore size from Millipore in France.

For secondary screening of tannase-producing fungus, the modified Czapek-Dox minimum medium was employed (Bradoo et al., 1997). The following components (g/L) were used to create it: 10 tannic acid; $NaNO_3$, 6; KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; KH_2PO_4, 1.52; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, pH 4.5 0.2; Cu $(NO_3)_2 \bullet 3H_2O$, 0.01. As specified in the preparation of the TAA medium, sterilization and pH correction were completed.

For the maintenance of fungal isolates, potato dextrose agar (PDA) supplemented with 0.01% tannic acid was utilized (Bajpai and Patil, 1996). The following components, g/l, were used to create it: agar 15, glucose 20, potatoes (200 g), infusion 0.1 tannic acid, and pH 5.6-0.2.

Isolation of tannase-producing fungi

With sterile distilled water, each soil sample was serially diluted (10^{-1} to 10^{-6}). Using the pour plate method, 1 mm from each dilution was plated into TAA medium. The plates were incubated at 30°C for 72 h under aerobic conditions. Selection and purification of fungi capable of growing and creating clearing zones around their colonies (Murugan et al., 2007). The acquired cultures were cultivated on PDA with 0.01% tannic acid supplementation, slants, and kept at 4°C (working cultures). Cultures of stock were kept in paraffin oil.

Screening and selection of tannase-producing fungal cultures

Using TAA plates in accordance with Bradoo et al. (1997) instructions, a primary screening for the highest tannase producers was completed (1996). The isolate was point injected onto the plates, and they were then incubated at 30°C. After 72 h of incubation, the diameter of clear zones (including the diameters of the colonies created as a result of the hydrolysis of tannic acid) around the fungal colonies was measured, then compared to determine the top tannase producers. Fungal cultures with strong tannase activity in the main screening were exposed to submerged fermentation for secondary screening (Batra and Saxena, 2005; Bradoo et al., 1997).

Each possible tannase-producing fungus's spore count was 5107, and 250 mL Erlenmeyer flasks containing 50 mL of sterilized modified Czapek-minimum Dox's medium (pH 4.5, 0.2) were used for inoculation. At the conclusion of the fermentation period, the extracellular tannase activity per flask was measured after cultures were grown at 30°C for 96 h.

Identification of tannase producing microorganisms

The fungal isolates were identified based on their morphological and microscopic characteristics. The molecular identification of the isolates up to species level is based on their ITS region carried out

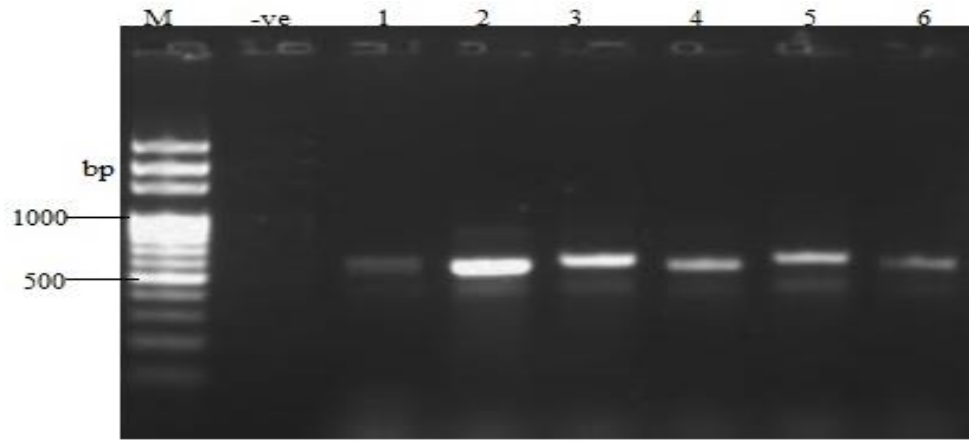

Figure 1. PCR amplication image of 18SrRNA gene bands of the fungi isolated from soil. Lane 1: DNA marker, Lane 2-6 are the 18SrRNA of the isolates.

by extracting their DNA using the Zymo Fungal/Bacteria using a DNA extraction kit as directed by the manufacturer. The ITS gene of the fungus was amplified by polymerase chain reaction using the primer pairs ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out using the Solis Biodyne 5XX Hot Firepol Blend Master mix according to the manufacturer's instructions (Figure 1).

Thermal cycling was done in an Eppendorf Vapoprotect thermal cycler (Nexus Series) for 35 amplification cycles, each lasting 30 s at 95°C, 1 min at 58°C, and 1 min 30 s at 72°C. A final extension phase lasting 10 min at 72°C was then performed. On a 1.5% agarose gel, the amplification product was separated, and electrophoresis was then done at 80 V for 1 h and 30 min. DNA bands were visible after electrophoresis, thanks to ethidium bromide staining. As a benchmark for DNA molecular weight, a 100 bp DNA ladder was employed. Exo sap was used to clean up the PCR products before they were delivered to Epoch Life science in the USA for Sanger sequencing.

Harvesting the enzyme and enzyme assay

Through Whatman No. 1 filter paper, the fermentation medium (Czapek-minimum Dox's medium) was purified. The resulting filtrate was used to measure extracellular tannase according to the described procedure by Libuchi et al. (1972). The reaction mixture was made by adding 2 mL of 0.3% (w/v) tannic acid in a 0.005 M citrate buffer (pH 5.5) solution to 0.5 mL of crude enzyme in various doses. To stop the enzyme reaction, 0.1 mL of the reaction mixture was removed from the whole system and 2 mL of an ethanol solution was added. After adding ethanol, the absorbance on the UV spectrophotometer was reported as t1 at 310 nm and t2 after 10 min of incubation at 37°C. The quantity of enzyme necessary to release 1M of gallic acid/min under specific circumstances is referred to as one unit of tannase activity. In U/mL units, enzyme activity was expressed.

Estimation of gallic acid

Using Bagpai and Patil's (1996) approach, the amount of gallic acid in the cultured broth was estimated. 1 mL of the culture supernatant was dissolved in 9 mL of citrate buffer at pH 5.5, and the absorbance was determined using UV spectrometric methods at 254.6 and 293.8 nm. The equation was used to determine the concentration:

Gallic acid (µg/mL) = $21.77(A_{254.6}) - 17.17(_{A293.8})$

Estimation of total tannin

The modified Price and Butt (1977) technique was used to calculate the waste's total tannin content. The reaction mixture was produced up to 10 mL with distilled water and contained the extract (0.5 mL), potassium ferric cyanide K3Fe(CN)6 (1%, 0.1 mL), and ferric chloride $FeCl_3$ (1%, 1 mL). Tannic acid was used as the standard to measure the absorbance at 720 nm. An extrapolation of the total tannin concentration was done using the tannic acid calibration curve (R2 = 0.9984).

Protein content determination

According to Lowry et al. (1951)'s description, the crude supernatant's protein content was estimated. 0.8 mL of distilled water was added to 0.2 mL of protein extract after it was measured into tubes. As a blank, distilled water (1.0 mL) was used, and a 100 g/mL Bovine Serum Albumin (BSA) standard curve was also built up. All of the tubes received 5.0 mL of alkaline solution containing 10 to 100 g/mL, which was added, carefully mixed, and left to stand for 10 min. After adding 0.5 mL of Folin-Ciocalteu solution, each test tube was given 30 min to settle before the optical density was measured at 280 nm in the spectrophotometer. Using data derived from the protein's standard calibration, the protein concentration was approximated.

Fermentation of agricultural waste

According to the approach used by Mohapatra et al. (2006), tannase was produced by the isolated organism by submerged fermentation of crude tannin from several agro-residues. The plant samples were collected aseptically in sample bags, cleaned, and dried in an oven at 60°C before fermentation. The samples were then processed via a grinder mixer until they were powdered. The powder was utilized as a source of crude tannins in submerged

Table 1. Microscopic and macroscopic features of fungal isolates.

Isolate	Macroscopic features	Microscopic features	Species identified
$PLA3_2$	Yellowish, cotton-like	Long, erect conidiospores, round shaped	*Penicillium* spp.
$CA6_2$	Black and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$CA6_1$	Yellowish, cotton-like	Long, erect conidiospores, round shaped	*Penicillium* spp.
$PLA3_1$	Brown center and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$PLA5_1$	Brownish-yellow and powdery like	Conidiospores, smooth walled and non-septate	*Aspergillus* spp.
$PLA6_1$	Yellow pink colonies creamy	Cylindrical to ovoid conidia, curved septate conidiospores	*Fusarium* spp.

Table 2. Identification of fungal isolates with ITS region of rRNA gene sequence.

Isolate	Species identified	Length (bp)	Identity (%)
$PLA3_2$	*Penicillium citrinum*	537	100
$CA6_2$	*Aspergillus niger*	575	99
$CA6_1$	*Penicillum citrnum*	523	91
$PLA3_1$	*Aspergillus niger*	570	94
$PLA5_1$	*Aspergillus vesicolor*	671	96
$PLA6_1$	*Fusarium equiseti*	570	92

fermentation and kept in a dry location in sterilized bottles at room temperature. The distilled water (200 mL) was combined with the powdered samples (50 g) and left at room temperature overnight. The mixture was simmered for 10 min after soaking. Crushed tannin was obtained from the filtered solution. After sterilization, the medium's pH was adjusted to 5.0. Crude tannin was fermented and submerged at 35°C in 250 mL Erlenmeyer flasks containing 50 mL medium and 1% (v/v) fresh inoculum. The enzyme was obtained from the cell-free fermented broth. We measured the dry weight of the biomass to track the organism's development in culture conditions (mg). By centrifuging the biomass, the biomass was separated, and the supernatant was used for tannase test.

RESULTS AND DISCUSSION

Soil inhabits diverse species of microorganisms including fungi and bacteria that have been predominantly used for the industrial production of enzymes. The biodiversity and variation of fungi isolated from soil depend on factors such as geographical locations, salinity, soil pH, moisture content, organic carbon, nitrogen sulfur, and potassium content affects (Sharma and Raju, 2013; Yu et al., 2007). Different fungi have been isolated and identified from soil including *Aspergillus, Penicillium*, and *Mucor* (Chandrashekar et al., 2014; Gaddeyya et al., 2012). In this study, tannase producing fungi were isolated from soil samples down tannin-rich plants including plantain, cocoyam, Malay apple, yam, pawpaw, cashew, almond, cassava, and neem. The fungal isolates were firstly identified to a genus level using morphological and microscopic features by considering the color of colonies formed at both sides, the top and reverse of the fungal cultures, and the shape of the spore-producing structures as shown in Table 1 (The suspected organism to the genus *Aspergillus*, *Fusarium,* and *Penicillium*). This method of fungal identification is limited to the family or genus level (Wang et al., 2016; Lutzoni et al., 2004). Therefore, molecular identification utilizing DNA barcoding and ITS region sequencing was done for authentication and identification down to the species level. One of the most crucial sequences is that of the ITS rDNA region and widely used instruments for identifying fungal species because they are widely dispersed, consistently functioning, and sufficiently preserved to offer a thorough understanding of evolutionary connections (Anderson and Parkin, 2007; Madigan et al., 2012). The sequence analysis of the ITS regions of the nuclear-encoded rDNA showed significant alignments of 90 to 100% with the isolated fungal species. The isolates were identified to be *Penicillium citrinum*, *Aspergillus niger* strain, *Penicillium citrinum* isolate K9, *A. niger* strain 7806F, *Fusarium equiseti* isolate GS-WW-F14-13, *Aspergillus versicolor* isolate 777, and *A. niger* strain SCSGAF0145 (Table 2). All the fungi isolated in this study have been previously reported to be tannase producers viz: *Aspergillus versicolor* (Batra and Saxena, 2005), *P. citrinum* (Lekha and Lonsane, 1997), *A. niger* (Wkil et al., 2020) except *F.equiseti*.

The identified fungi presented in Table 2 are good tannase producers based on the results presented in Table 3. The diameter of clear zones around the isolated colonies depicts their ability to degrade tannic acid to gallic acid and glucose. The *Aspergillus* strains produced higher diameter of clearance zone compared to *Fusarium* and *Penicillium* strains. This is substantiated considering their biomass weight, gallic acid concentration and tannase activity in the fermentation broth by utilizing

Table 3. Primary and secondary screening of isolates.

Organism	Clear zone diameter (mm)	Biomass weight (g)	Gallic acid concentration (µg/mL)	Tannase activity (IU/mL)
Penicillium citrinum	18 ± 0.1	0.11±0.01	7.62±0.18	3.24±0.14
Aspergillus niger	26 ± 0.06	0.21±0.01	8.02±0.10	4.83±0.22
Penicillum citrnum	15 ± 0.1	0.07±0.02	7.98±0.15	3.92±0.12
Aspergillus niger	25 ± 0.06	0.19±0.02	8.16±0.31	5.83±0.18
Aspergillus vesicolor	25 ± 0.1	0.13±0.01	7.98±0.23	4.77±0.02
Fusarium equiseti	17 ± 0.12	0.04±0.04	7.50±0.33	2.64±0.33

Values are expressed as Mean±SD of triple determinants.

Table 4. Concentration of tannin in plant extract.

Plant sample	Concentration (mg/mL)
Pineapple	10.336 ± 0.12
Acacia	7. 785 ± 0.08

Values are expressed as Mean±SD of triple determinants.

Table 5. Concentration of tannin in plant extract.

Organism	Substrate	Tannin concentration (mg/mL)	Biodegradation (%) of tannins	Gallic acid concentration (mg/mL)	Protein concentration (mg/mL)	Tannase activity (IU/mL)
A. versicolor	Pineapple	5.03±0.08	51.94±0.31	0.49±0.17	12.75±0.35	3.57±0.41
	Acacia	1.53±0.05	80.98±1.88	9.42±0.39	26.09±0.75	22.49±1.17
F. equiseti	Pineapple	4.43±0.41	55.87±3.95	3.75±0.27	10.70±0.67	1.53±0.17
	Acacia	2.96±0.32	63.46±1.26	4.58±0.48	21.34±1.14	12.99±1.17
P. citrinum	Pineapple	3.57±0.21	67.88±0.92	8.26±0.43	24.65±0.70	5.61±0.53
	Acacia	1.83±0.04	77.28±1.14	8.57±0.31	21.06±0.38	16.23±0.66

Values are expressed as Mean±SD of triple determinants

tannin acid as their sole carbon source. Pinto et al. (2001) reported a correlation between colony diameter (zone of clearance) and tannase production using *A. niger* strains. Thus, these isolates can be used as a source of the enzyme for tannin bioconversion process (Shete and Chitanand, 2015).

In this study, the ability of *A. versicolor*, *F. equiseti* and *P. citrinum* to secrete tannase using agricultural wastes-pineapple peel and acacia nuts as substrate was assessed. The total tannin content of the plant material was presented in Table 4. The tannin concentration of pineapple peel was higher than acacia nuts. The choice of a substrate for enzyme and subsequent product formation by fermentation depends on the cost, availability, and suitability of the substrate for obtaining the desired product of fermentation, and thus, requires screening of several agro-industrial residues (Pandey et al., 1999). Acacia trees are found abundantly within the premises of Lagos State Polytechnic, Ikorodu and their nuts have constituted important environmental pollutants, thus, its selection for this project. The tannin concentration in each fermentation broth was determined. It was observed that there was a decrease in tannin concentration in all the fermentation flasks but *A. versicolor* showed the least tannin concentration using acacia nut extract as substrate. The reduction in tannin concentration implies the degradation of tannin by the organisms. The utilization of the agricultural waste was substantiated by the gallic acid concentration in each fermentation flask.

The results of biomass weight (Table 5) show that the organisms used in this study *A. versicolor*, *F. equiseti* and

*P. citrinum*a utilized the tannin extract obtained from the agricultural waste for growth. *A. versicolor* had the highest biomass weight for acacia as substrate while *F. equiseti* had the least biomass weight for both pineapple and acacia extract. This shows that the fungi can be grown abundantly on acacia nuts for the production of single-cell protein. Single cell protein are dried cell mass of fungi, molds, and bacteria used as protein supplements in animal and human feed to augment their diet (Yalcın et al., 2009).

Tannase, an inducible extra-cellular enzyme produced by several animals, plants, and microbes, has wide application in tannery, alcohol industry, pharmaceuticals and beverage industries. Many researchers have reported the secretion of tannase by fungi using various agricultural materials as substrate (Reges de Sena et al., 2014). Tannase activity was highest in acacia nut extract produced by *A. versicolor* (21.146 U/mL) by submerged fermentation.

Conclusion

The present study revealed the presence of fungi in various soil samples with the ability to secrete tannase. The fungi strains identified are *A. versicolor*, *F. equiseti* and *P. citrinum* and showed good activity using pineapple peel and acacia nuts as substrates evident in the reduction in tannin concentration of various substrates and gallic acid concentration, however, *A. versicolor* showed higher activity compared to the other fungal strains used in the study. In conclusion, the isolated fungal strain is good tannase producers which can be explored for industrial processes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Identification of antagonistic bacterium strain and biocontrol effects on ginseng root rot disease

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From healthy mountain-cultivated ginseng leaves, 45 endophytic bacteria strains were isolated. Antagonistic bacterium strain FS17 was screened with the confront culture method, and identified as *Bacillus subtilis* based on morphological characteristic and 16S rRNA sequence analysis. Growth rate method was used to determine that the fermentation broth of FS17 had inhibitory effect on 10 plant pathogenic fungi, and the inhibition rate ranged from 46.83 to 93.25%. The fermentation broth of strain FS17 with lower disease incidence and lesion diameter had better control effects with no significant difference compared with that of carbendazim. This study suggests that strain FS17 showed strong inhibition effect and wide antagonistic spectrum, and could be useful to effectively control *Cylindrocarpon destructans* causing ginseng root rot.

Key words: Ginseng root rot, antagonistic bacterium, *Bacillus subtilis*, biocontrol.

INTRODUCTION

Ginseng root rot is caused by *Cylindrocarpon destructans*, this disease is a destructive disease in the process of ginseng planting, the mortality rate of more than half (Li et al., 2022). Ginseng root rot is a kind of soil-borne diseases, severely impacts the quality of ginseng. Most infections occur during the rainy and winter season, because these seasons are highly ideal for fungal mycelial growth, which leads to spore invasion of the plants with initial indications of leaf loss and decreased root development in the plant (Kim et al., 2017). Ginseng seedlings is invaded, which symptoms are not obvious in the early stage, then the leaves turn yellow slowly in the late, finally lead to wilt, which take is greatly harmful to the ginseng production (Park et al., 2017).

At present, a variety of chemical pesticide such as carbendazim, thiophanate-methyl, fludioxonil, myclobutanil, hymexazol and pyrametostrobin and so on are widely used in the prevention and control of ginseng root rot (Sun et al., 2020). However, with the application of chemical pesticides, plant pathogens will develop resistance, and cause severe threat to the environment security (Cameron and Sarojini, 2014). Therefore, the biological control of ginseng root rot is the most ideal method. Gou et al. (2015) screened *Bacillus amyloliquefaciens* HB - 3 from ginseng rhizosphere soil; it can significantly inhibit the growth of ginseng root rot

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fungus; Wang et al. (2019) understand that *B. amyloliquefaciens* FS6 can stable colonize ginseng plant body and conduction, and can to a certain extent induced disease resistance of ginseng. The endophytic bacteria mainly live in plant tissues, they can be colonized long-term in the plant body, and it is not susceptible to the influence of the external environment condition. Endophytic bacteria and pathogens is the relationship of mutual benefit, which is a kind of important bio-control resources (Kim et al., 2020). This study was based on isolating endophytic bacteria from the leaves of *Panax ginseng* and screening antagonistic bacteria strains against ginseng root rot. Strain morphological and molecular identification were completed. The strain FS17 was identified as *Bacillus subtilis,* which had the very good biological control effect on ginseng root rot disease.

MATERIALS AND METHODS

Strains

Cylindrocarpon destructans, Fusarium solani, Fusarium graminearum, Fusarium semitectum, Pestalotiopsis paeoniicola, Trichothecium roseum, Fusarium proliferatum, Fusarium oxysporum, Alternaria panax, Fusarium equiseti and *Cladosporium cladosporioides*, were obtained from the plant pathology lab, Institute of Special Animal and Plant Sciences of CAAS, China. Each pathogen was recultured in a Petri dish containing potato dextrose agar (PDA) and incubated at 25°C for 5 day stored at 4°C until use.

Screening of antagonistic bacteria

The antagonist activity of bacterial isolates was tested against *C. destructans*, using the dual culture technique described by Zhou et al. (2021). Bacterial strains were streaked at the edges of Petri plates containing Potato Dextrose Agar medium and, after 48 h of incubation at 28°C, a 6mm mycelial plug of pathogenic fungus was placed in the centre of each plate. The plates were then incubated at 28°C for 5 days. All experiments were performed in triplicate and repeated three times based on the inhibitory effect of bacteriostatic ring size screening of strain (Zhou et al., 2021).

Morphological identification

The morphological characteristics of antagonistic strain were observed after incubation on BPA (The reagent company of Shiao, China) at 28°C for 3 day, and identified using light microscopy according to the morphological characteristics.

16 s rRNA sequence analysis

Using 50 µL amplification reaction system: Taq mixture 25 µL, universal primers 27F(5′-AGAGTTTGATCCTGGCTCAG-3′) 2 µL and 1492R(5′-GGTTACCTTGTTACGACTT-3′) 2 µL, DNA template 2 2 µL, ddH_2O 19 2 µL. Amplification conditions for: 95°C in advance 4 min 55°C 94°C modified 30 s, annealing 45 s, 72°C 1 min, 30 cycle; finally extended under 72°C 8 min, eventually check response (Kim et al., 2019). Amplified PCR products were cloned and sequenced according to the Pan's protocol, and nucleic acid identified was determined using NCBI BLAST (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was conducted using maximum likelihood in MEGA 5.10 and then the topology of the phylogenetic tree was evaluated by 1,000 resamplings.

Bacteriostatic spectrum determination of strain FS17

The antagonist bacteria strains were inoculated in LB medium under the condition of 28°C, 220 r·min^{-1} for 24 h. Liquid was fermented at 4°C, 10000 r·min^{-1} after centrifugation for 10 min, using 0.45 µm membrane filtration. Sterile fermentation liquor made from fermented liquid of 1, 3, 5, 7 and 9% concentrations was cooled to 40 to 45°C, respectively with melt blending, PDA medium in tablet, with PDA medium without sterile fermentation liquor as a control. Six kinds of pathogenic fungi cake (5 mm in diameter), was developed in 7 day in the middle plate at 25°C, and mycelium under the condition of constant temperature was cultured for 7 day. Computation formula of inhibition rate is as follows:

Inhibition rate (%) = (controlled colony diameter - processing colony diameter) / (controlled colony diameter - 5) ×100.

Control effect of strain FS17 against *F. solani*

Health ginseng roots were washed with tap water, reoccupy 2% NaClO soaking treatment 3 min, and then to dry after washing three times with sterile water. The wound (size 2 × 2 mm) was made using sterilized needle on the root surface. The roots were inoculated with 20 µL strain FS17 fermented liquid (10^8 CFU/mL). After 4 h, the spore suspension of *C. destructans* were shaken on the roots, with water as a control, carbendazim (1000 µg/mL) as the reagent control, each dealing with a repeat 3 times. At 25°C, relative humidity of 95% in the constant temperature incubator, respectively in the 3 and 5 days after statistical disease incidence, and to measure disease spot diameter (Wang et al., 2017).

Data statistics and analysis

Data were compiled using EXCEL (Microsoft). At least three independent experiments were performed in each case. The values were represented as mean ± SD of three replicates for each treatment. Statistical significance was analysed with Student's *t*-test and analysis of variance followed by Tukey's post hoc test (SPSS v. 13.0; SPSS Inc).

RESULTS

Screening of antagonistic bacteria strains

A total of 45 endophytic bacteria strains were isolated from the leaves of forest ginseng. Eight of these showed prominent antagonistic activities against *C. destructans in vitro.* Among them, 17 strains had bacteriostasis effect of ginseng root rot; strain FS-17 had an 21 mm of inhibition zone against *C. destructans* in the dual-culture test (Figure 1).

Morphological identification of antagonistic bacteria

The colonies of FS-17 strain was white, rough surface

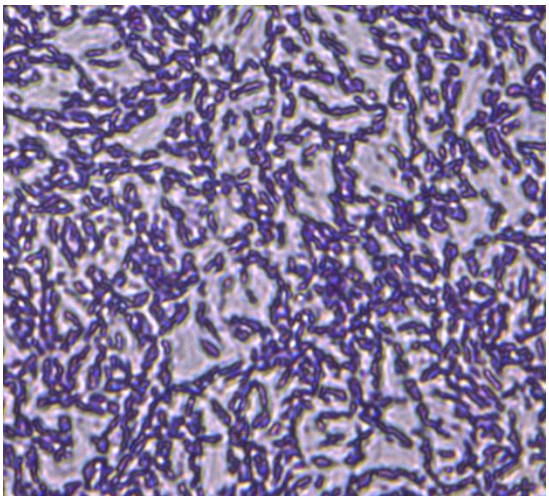

Figure 1. Morphological characteristics of strain FS17. A: Morphological characteristics of strain 17 on BPA culture medium B: Morphological characteristics of strain 17 under electron microscope.

Table 1. Physiological and biochemical characteristics of strain FS17.

Item	Result
Arabinose	-
Gelatin hydrolysis	+
Nitrate reduction	+
Methyl red	+
Glycerinum	+
Benzpyrole	+
氧化酶试剂	+
Fructose	+
Sucrose	+
Galactose	-
Voges-Proskauer test	+
Dulcitol	-
Mannitol	-
Citrate utilization	+
Malonate utilization	+
Catalase	+
Glucose	+

was opaque on BPA medium, 24 h, 37°C constant temperature culture. The FS-17 strain was rod, produced spores, born in or close to, size 0.7- 0.8 μm×2.0-2. 5 μm, Gram positive reaction (Figure 1B). The physiological and biochemical characters of FS-17 strain are shown in Table 1. A preliminary test showed that the strain FS-17 was identified as *Bacillus* species according to morphological characteristics combined with the physiological and biochemical test results.

Molecular identification of antagonistic bacteria

PCR products of 1500 bp were obtained from amplification of the 16S rRNA of the genomic RNA of strain FS-17. Sequence analysis showed that strain FS-

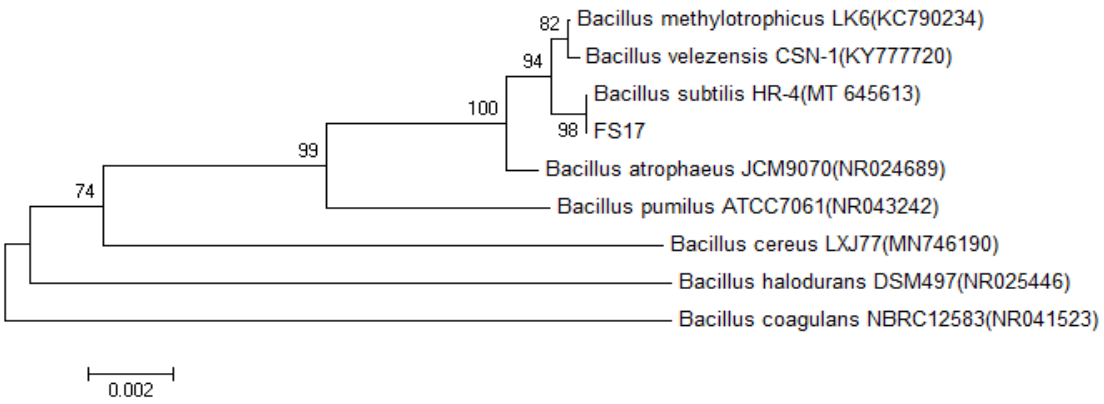

Figure 2. Phylogenetic tree of strain FS17 based on the 16S rRNA.

Table 2. Inhibition effects of fermentation broth from strain FS17 on plant pathogenic fungi.

Pathogen	Inhibition rate of different concentration (%)				
	1%	3%	5%	7%	9%
F. graminearum	5.17±3.12^{ab}	7.35±1.08^{c}	26.17±1.43^{d}	45.37±3.94	48.02±2.97^{d}
F. semitectum	3.82±0.53^{ab}	22.17±5.12^{abc}	25.79±3.16^{d}	35.24±5.24^{d}	46.83±2.58^{d}
F. solani	5.74±4.01^{ab}	11.32±9.14^{c}	34.51±8.53^{cd}	44.15±3.64^{d}	49.48±6.62^{d}
P. paeoniicola	0.61±1.02^{b}	6.35±7.61^{c}	52.42±9.36^{bc}	90.27±5.37^{a}	90.27±5.58^{a}
T. roseum	12.37±16.22^{a}	33.72±2.28^{c}	78.47±5.27^{a}	90.58±2.37^{a}	91.55±3.82^{a}
F. proliferatum	1.73±0.97^{ab}	4.58±2.21^{c}	2.94±1.53^{d}	72.14±4.02^{bc}	79.52±1.05^{ab}
F. oxysporum	3.97±3.58^{ab}	51.59±8.13^{a}	63.27±16.48^{ab}	67.49±4.13^{bc}	76.14±5.94^{b}
A. panax	6.93±6.52^{ab}	41.46±11.57^{ab}	73.25±7.98^{a}	80.23±3.12^{ab}	80.59±3.12^{ab}
F.equiseti	7.35±2.38^{ab}	12.97±2.47^{ab}	25.53±1.58^{c}	46.42±3.19^{d}	56.48±2.58^{d}
C. cladosporioides	13.27±1.49^{a}	43.84±2.91^{c}	80.32±1.74^{a}	91.23±2.41^{a}	93.25±3.04^{a}

17 shared 100% identity with a number of *B. subtilis* in the NCBI database (Accession No. MT645613). Phylogenetic tree was constructed by using 16S rRNA sequences, and it clearly showed that strain FS-17 is clustered with members of the genus *Bacillus* (Figure 2). Strain FS-17 was identified as *B. subtilis* based on the results of the 16S rRNA sequence analysis and the morphological characterization.

Bacteriostatic spectrum determination of strain FS17

Bacteriostatic spectrum test results showed that different concentrations of strains fermented liquid of FS-17 of 10 kinds of plant pathogenic fungi have certain inhibitory effect. Its bacteriostatic spectrum ranges with the change of the fermented liquid concentration, the inhibition rate of pathogens was changing. Among them, when the fermented liquid concentration was 9%, bacteriostatic rate of *F. solani* was highest, at 93.25%, bacteriostatic rate of *F. semitectum* was the lowest, at 46.83%, bacteriostatic rate of the other 8 kinds of pathogens was between 48.02 and 91.55% (Table 2).

Control effect of strain FS17 against *C. destructans*

The test results showed that the incidence rate and disease spot diameter of strain FS17 fermented liquid and carbendazim were significantly than water control. The control effect of strain FS17 fermented liquid against *C. destructans* was 40.15 and 65.18%, respectively after dealing with 3 and 5 days. And carbendazim processing control effect was 48.32 and 67.29%, respectively. The control effect of strain FS17 fermented liquid against *C. destructans* was slightly lower than contrast agents carbendazim (Table 3).

DISCUSSION

At present, carbendazim, thiophanate-methyl, fludioxonil, myclobutanil, hymexazol, pyrametostrobin, etc., chemical pesticides were used to prevent and control ginseng root

Table 3. Control effects of strain FS17 on ginseng root rot.

Treatment	3 days		5 days	
	Colony diameter (mm)	Control effect (%)	Colony Diameter (mm)	Control effect (%)
Water	5.21±0.43[a]	-	11.63±1.43[a]	-
Strain FS17	3.48±0.29[b]	40.15[a]	4.02±0.61[b]	65.18[a]
Carbendazim	3.12±0.94[b]	48.32[a]	3.47±0.53[b]	67.29[a]

rot disease. But studies have shown that with long-term use of chemical pesticides, pathogen will develop resistance (Saito et al., 2016). Therefore, there is need to seek new methods of prevention and control of ginseng root rot, to slow down the occurrence of the pathogenic bacteria drug resistance and its life cycle in plant tissues, endophytic bacteria in plant body can colonize in long-term stability, and is not susceptible to the influence of the external environment condition. Endogenous bacteria and pathogen have mutual reciprocity and benefit; they are a kind of important resources (Aly et al., 2011). Report has it that *B. amyloliquefaciens*, *Bacillus methylotrophicus, Paenibacillus polymyxa* and *Enterobacter cloacae* can be used for ginseng disease prevention and control (Olamide et al., 2019; Durairaj et al., 2018; Wang et al., 2016; Jiang et al., 2013), and *B. subtilis* can be used for the prevention, and treatment of ginseng root rot has not been reported.

Endophytic bacillus is a kind of non-pathogenic bacteria that can produce spores and a variety of antibacterial substances, such as antibiotics and antimicrobial proteins. Bacillus resistance is strong, fast in reproduction, very easy to survive in the environment, colonization is a kind of important resources of bio-control bacteria, it has wide application in the green plant disease prevention and control. In this study, a strain isolated from the leaves of *P. ginseng* has a very good inhibitory effect to ginseng mould bacterium, FS17 endophytic bacteria identified the strain for *B. subtilis*, strain bacteriostatic spectrum is more extensive, FS17 to 10 kinds of common plant pathogenic fungi are highly bacteriostasis. The control the effect of FS17 strain against ginseng root rot was remarkable. Disease spot diameter was much smaller than control, comparative with the control effect of carbendazim. Therefore, the FS17 strain can be used for prevention and control ginseng root rot.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Inoculation effectiveness of native and exotic *Bradyrhizobium* species strains in a Senegalese agricultural soil: A comparison on modern and traditional peanut (*Arachis hypogaea* L.) cultivars

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Peanut is a key component of Senegal's predominantly cereal-based farming systems, but its production is challenged by low soil fertility. Rhizobial inoculation is a promising strategy to improve crop yield and reduce the use of chemical nitrogen fertilizers. The aim of this study was to isolate the most specific and effective bradyrhizobial strain for peanut, and to determine the degree of variability in the response of peanut cultivars to inoculation. The seeds of five cultivars: *55-437, Fleur 11, Sunu Gaal, Amoul* Morom and *Essamaay* were inoculated individually with ten bradyrhizobial strains (LMG9283 and USDA3187, which are the reference strains; ISRA400, ISRA453, ISRA454, ISRA519, ISRA534 and ISRA538, isolated from *Fleur 11* in Senegal, and ORS3640 and ORS3644, isolated from herbaceous species that are commonly found in Senegalese farmers' fields). The plants were grown under greenhouse conditions in a mixture of sandy soil and vermiculite (1/1, v/v). The results obtained in terms of nodule formation, plant growth and yield parameters showed a positive effect of bradyrhizobial inoculation. However, these data indicated that the response of peanut to inoculation was cultivar dependent, with the traditional cultivars *55-437* and *Fleur 11* showing the greatest increase in plant growth and yield parameters. Our results also highlighted the need for cultivar-specific selection of *Bradyrhizobium* to improve inoculation success in peanut, with the indigenous isolates being specifically more effective than the reference strains. According to this study, it would be beneficial to promote the use of native isolates that perform well with the peanut cultivars studied.

Key words: Peanut (*Arachis hypogaea*), inoculation, indigenous and exotic *Bradyrhizobium* strains, nodulation, growth, yield parameters.

INTRODUCTION

The peanut, also known as groundnut (*Arachis hypogaea* L.), is an important grain legume grown in the tropics and

subtropics, including sub-Saharan Africa, and consumed worldwide for human and animal feeding (Noba et al., 2014). In Senegal, peanut has been a cash crop for over a century, contributing to 60% of the country's agricultural gross domestic product and approximately 80% of its export earnings (Sene et al., 2010; Noba et al., 2014). It is the main oil-producing crop and the four oil factories established in the country formed the backbone of the national industrial fabric. After a long period of decline, peanut yields have increased in the last five years. However, the factors that determine these increases, that is, soil fertility, have steadily deteriorated, with a reduction in fallow land and low levels of fertilizer use (Sene et al., 2010, 2023). Various agricultural practices, including the use of urea, have been adopted to boost yields and mitigate food shortages. However, the high cost of chemical nitrogen fertilizer and the necessity for sustainable alternative sources has increased the strategic importance of microbial inoculation.

Microbial inoculation is a promising strategy to improve crop yields and minimize dependence on chemical fertilizers, thereby fostering environmentally friendly agriculture (Kahindi et al., 1997; Garg et al., 2018; Itelima et al., 2018; Sene et al., 2023). Among these microbial communities, soil bacteria, collectively referred to as rhizobia, hold a pivotal role in improving crop production (Giller, 2001). The demand for rhizobial inoculants is therefore growing, driven by the need for sustainable and environmentally friendly agricultural practices and safer and healthier food (Lesueur et al., 2016; Mohanty and Swain, 2018; Sene et al., 2021, 2023).

Rhizobia are symbiotic bacteria that induce the formation of new organs called nodules on the roots of certain legume hosts, within which the bacteria multiply, differentiate into bacteroids and subsequently convert the atmospheric nitrogen into ammonia (Peoples et al., 1995; Kahindi et al., 1997; Giller, 2001). The rhizobia-legume symbiosis is one of the most important nitrogen-fixing systems (Kahindi et al., 1997). The isolation and selection of elite rhizobial strains is very important because the effective rhizobial strains can be used as inoculants for effective nodulation (Dudeja and Khurana, 1988; Dhery and Dreyfus, 1991; Lanier et al., 2005; Bogino et al., 2006; Zaiya et al., 2018). Inoculation can provide sufficient numbers of viable and effective rhizobia to facilitate rapid root nodulation and ultimately to achieve optimum yields. Rhizobial inoculants offer an alternative to industrial nitrogen fertilizers and a means of preserving or improving soil fertility (Peoples et al., 1995; Alves et al., 2003; Chalk et al., 2006). Despite their potential, the commercialization of microbial inoculants has lagged behind the expectations in West Africa, and successful establishment of legume crops in these countries requires an effective symbiotic association between elite strains and compatible host plants (Lesueur et al., 2016). Like many other legumes, peanut has the ability to form a mutualistic symbiosis with rhizobia. For this symbiosis to be successful, there must be a sufficient quantity of fresh, vigorous bacteria ready to enter the roots and multiply rapidly. This symbiotic partnership serves as the most efficient and effective method of supplying nitrogen to the leguminous crops, especially when the soil is void of the specific rhizobial agents. In Senegal, peanut is so far considered to be nodulated by the genus *Bradyrhizobium* (Sene et al., 2010; Zaiya et al., 2018), the so-called slow-growing rhizobia. However, selected bradyrhizobial strains often fail to compete with indigenous soil rhizobia in Senegalese soils and do not increase nodulation. Their competitive ability is an important factor in determining their success (Sene et al., 2010). Furthermore, some authors reported that modern high-yielding and traditional cultivars differ in their response to microbial inoculation (Chen et al., 2003; Meghvansi et al., 2008; Argaw, 2017). This suggests the need for cultivar-specific rhizobial selection prior to inoculum formulation. Therefore, the present study was undertaken to isolate the most specific and effective *Bradyrhizobium* species inoculants for five modern and traditional Senegalese peanut cultivars and to use elite strains as inoculants. Our hypothesis asserts that the peanut's response to rhizobial inoculation would vary between cultivars and that this variability would differ between modern and traditional cultivars.

MATERIALS AND METHODS

Plant

Five local peanut (*A. hypogaea* L.) cultivars kindly obtained from the Centre National de Recherche Agronomique (CNRA) in Bambey, Senegal, were used in this experiment. These cultivars were selected on the basis of the taste desired by the local population and their characteristics shown in Table 1.

Bradyrhizobial materials

The bradyrhizobial strains used in this study are from the collection of the Laboratoire Commun de Microbiologie (LCM) IRD/ISRA/UCAD, Dakar, Senegal. Six of them (ISRA 400, ISRA453, ISRA454, ISRA519, ISRA534 and ISRA538) are indigenous bradyrhizobia isolated from the peanut cultivar *Fleur* 11 grown in soils sampled from the Senegalese peanut production basin (Zaiya et al., 2018). The two strains ORS3640 and ORS3644 are also indigenous and were isolated from herbaceous species that coexist with peanut crops (Sene et al., 2012, 2013). In this experiment, they were tested against two reference bradyrhizobial strains (USDA3187 and LMG9283) (Castro et al., 1999; Sene et al., 2010).

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Table 1. Characteristics of the peanut cultivars used in the study.

Cultivar	Type	Growth habit	Growth cycle (days)	Registration in Senegal
Fleur 11	Spanish	Erect	90	Traditional, since 1955
55-437	Spanish	Erect	90	Traditional, since 1993
Sunu Gaal	Spanish	Erect	95	New, since 2017
Essamay	Virginia	Semi-erect	105	New, since 2017
Amoul Morom	Virginia	Semi-erect	120	New, since 2017

Greenhouse experimental design

The experiment was set up in the greenhouse (Bel Air Experimental Station, 14°44'N, 17°30'W in Dakar) using a non-sterile soil from Sangalkam, 30 km east of Dakar mixed with sterilized vermiculite at 120°C for 20 min (1:1, v/v). This soil has a pH of 6.5 with 58.15, 32.8 and 3.6% of sand, loam and clay, respectively and contains 0.06% total nitrogen (N), 0.54% total carbon (C), 39 mg phosphorus (P) kg^{-1} total P, 4.8 mg P kg^{-1} available P. It was sieved (< 1 mm), homogenized and used to fill up the pots. Seeds of selected cultivars (Table 1) of peanut were first surface sterilized (to avoid seed-borne diseases) with 5% sodium hypochlorite (NaOCl) for 5 min, 70% ethanol for 3 min and thoroughly rinsed with sterile distilled water. The seeds were then placed on Petri dishes containing moist filter paper for germination under sterile conditions and kept in the dark at 25°C. The germinated seeds were manually transplanted into 1.5 L plastic pots disinfected with a solution containing 1.81% of calcium hypochlorite and filled with the substrate to a depth of 2 to 3 cm. Two germinated seeds were planted in each pot. The plants were thinned on the 3rd day after planting to one plant per pot. The pots were arranged in randomized blocks, with a single inoculation and five replications. The pots were placed at 10 and 40 cm spacing within and between rows for the cultivars *Fleur 11*, *55-437* and *Sunu Gaal*. The distance between the pots was 10 and 60 cm for the cultivars *Amoul Morom* and *Essamay.* The plants were grown for 65 days under greenhouse conditions (temperature of 27-35°C, relative humidity of 70-80% and 12 h of light) and were watered every two days with chlorine treated tap water without added nutrients.

Inoculant preparation and inoculation

The greenhouse experiment consisted of 11 treatments: eight with application of indigenous bradyrhizobial inoculants compared with two reference strains, and a negative control without inoculation for each cultivar. The inoculants were prepared as follows: each *Bradyrhizobium* strain was grown in 250 mL Erlenmeyer flasks containing 100 mL yeast extract-mannitol (YEM) medium (Vincent, 1970) for 3 days at 28°C with rotary shaking at 150 rpm. Five milliliters (containing 10^8 cells mL^{-1}) of the bacterial culture at its logarithmic growth stage were used to inoculate the plants. The inoculants were applied directly to the soil surface at the base of the stem five days after emergence to ensure that the bacteria reached the roots. Treatments without bradyrhizobial inoculum received 5 mL of autoclaved inoculum in order to avoid differences in soil nutrient content associated with the addition of rhizobial inoculum.

Collection of growth and yield variables

Data on growth variables (plant height and number of branches) and leaf chlorophyll content for each cultivar were collected at flowering [30 days after planting (DAP)], pod filling (45 DAP) and pod maturity periods (60 DAP). Plant height (cm) was measured with a ruler from the base of the stem to the apex, while the number of branches was counted manually. Leaf chlorophyll content was estimated at 30, 45 and 65 DAP using a SPAD-502Plus chlorophyll meter (Konica-Minolta). At harvest, whole peanut plants were uprooted. The soil adhering to the roots was removed under running tap water and the nodules were picked and counted. The pods were manually stripped from the plants to record the yield components. For each cultivar, above-ground and root biomass, root colonization (number and biomass of nodules) and the yield attributes (number of pods per plant, pod weight) were determined. Above-ground and root biomass, nodule weight and the yield attributes were determined by weighing sample parts after over-drying to constant weight at 65°C.

Data analyses

All data were tested for normality and homogeneity using the Shapiro-Wilk and Levene tests, respectively. Data for plant growth and yield parameters were statistically analyzed using univariate analysis with one-way analysis of variance (ANOVA) using the R software v3.4.4 (R Core Team, 2020). Significantly different means and standard deviation were separated using the Tukey (HSD) test at the 5% probability threshold.

RESULTS

Plant nodulation

The results showed that there was a significant difference ($p < 0.05$) in nodule number and nodule biomass between the inoculated and uninoculated treatments for all cultivars (Table 2). Irrespective of the peanut cultivar, plants in the *Bradyrhizobium* strain ISRA519 treatment were more nodulated than the other inoculated strains and the uninoculated plants, indicating that this strain had a high nodule occupancy capacity. However, the nodulation was more pronounced in some cultivars rather than in others. In particular, in the traditional short-cycle peanut cultivars *55-437* and *Fleur 11* and in the modern short-cycle cultivar *Sunu Gaal*, the ISRA519 strain had the highest nodule number and nodule dry weight compared to the modern long-cycle cultivars *Amoul Morom* and *Essamay*.

In the *Amoul Morom* cultivar, inoculation with the indigenous strains ISRA 453, ISRA519, ISRA534 and ISRA538, isolated from the *Fleur 11* cultivar significantly increased the number of nodules. Inoculation with ORS3644, isolated from *M. atropurpureum*, also increased nodule number with strain LMG9283, also

Table 2. Nodulation (number and biomass of nodules) of five peanut cultivars (*A. hypogaea*) under single inoculation with *Bradyrhizobium* sp. 65 days after planting.

Treatments	Peanut cultivars									
	Amoul Morom		***Essamaye***		***55-437***		***Fleur11***		***Sunu Gaal***	
	NNum	***NDW (g)***	***NNum***	***NDW (g)***	***NNum***	***NDW (g)***	***NNum***	***NDW (g)***	***NNum***	*NDW (g)*
ISRA400	43.0±6.22^{f}	0.04±0.01^{ab}	63.0±10.7^{cd}	0.06±0.01^{abc}	50.8±16.8^{c}	0.04±0.01^{b}	74.0±5.77^{c}	0.04±0.01^{b}	111±8.37^{b}	0.07±0.01^{b}
ISRA453	87.5±12.7^{bc}	0.05±0.01^{ab}	81.0±6.38^{bc}	0.05±0.01^{abc}	47.0±1.83^{c}	0.05±0.02^{ab}	110±16.1^{bc}	0.06±0.01^{ab}	68.5±3.70^{b}	0.06±0.02^{b}
ISRA454	60.0±16.1^{def}	0.05±0.01^{ab}	91.0±8.25^{ab}	0.04±0.01^{abc}	26.3±2.22^{c}	0.04±0.01^{b}	112±13.0^{bc}	0.06±0.01^{ab}	60.0±6.16^{b}	0.05±0.01^{b}
ISRA519	133.0±6.08^{a}	0.06±0.01^{a}	111 ±4.43^{a}	0.05±0.01^{abc}	469±80.8^{a}	0.09±0.06^{a}	306±64.2^{a}	0.07±0.01^{a}	477±94.0^{a}	0.20±0.03^{a}
ISRA534	114.0±6.81^{ab}	0.06±0.02^{ab}	92.0±4.76^{ab}	0.03±0.01^{c}	126±3.79^{b}	0.05±0.01^{ab}	136±10.0^{b}	0.05±0.01^{ab}	122±14.2^{b}	0.05±0.01^{b}
ISRA538	90.0±12.3^{bc}	0.06±0.01^{ab}	68.5±14.1^{bcd}	0.04±0.01^{abc}	66.3±10.4^{bc}	0.05±0.01^{ab}	75.0±3.5^{c}	0.05±0.01^{ab}	81.3±15.9^{b}	0.05±0.01^{b}
ORS3640	53.8±14.7^{ef}	0.05±0.01^{ab}	82.5±15.0^{bc}	0.05±0.02^{abc}	55.5±5.20^{c}	0.06±0.01^{ab}	121±27.4^{bc}	0.04±0.00^{b}	108±4.97^{b}	0.05±0.01^{b}
ORS3644	72.3±7.76^{cde}	0.04±0.01^{ab}	90.0±10.6^{abc}	0.06±0.01^{abc}	64.5±21.8^{bc}	0.03±0.01^{b}	111±13.2^{bc}	0.04±0.01^{b}	109±15.9^{b}	0.04±0.01^{b}
LMG9283	81.5±12.8^{cd}	0.04±0.01^{ab}	75.3±21.2^{bcd}	0.05±0.02^{abc}	64.5±4.51^{bc}	0.03±0.01^{b}	88.8±6.50^{bc}	0.04±0.01^{b}	89.5±15.2^{b}	0.06±0.02^{b}
USD3187	65.3±7.09^{cdef}	0.05±0.00^{ab}	64.8±4.57^{bcd}	0.06±0.01^{ab}	59.8±20.3^{c}	0.04±0.01^{b}	84.0±11.6^{bc}	0.05±0.01^{ab}	49.0±9.06^{b}	0.05±0.01^{b}
Control	45.3±3.77^{f}	0.04±0.01^{b}	48.0±12.4^{d}	0.06±0.01^{a}	48.75±2.06^{c}	0.02±0.01^{b}	73.5±7.94^{c}	0.05±0.01^{ab}	69.8±8.77^{b}	0.06±0.01^{b}
Mea±sd	76.3±12.8	0.05±0.01	78.9±19.6	0.05±0.01	94.5±25.0	0.04±0.03	117±6.7	0.05±0.01	123±16.9	0.07±0.04
CV (%)	13.921	21.098	14.413	22.319	28.319	16.608	19.899	18.749	24.821	20.529
pValue	1.39 e^{-12}***	0.013*	3.47e^{-07}***	0.004**	<2 e^{-16}***	0.004**	4.16 e^{-14}***	0.002**	<2 e^{-16}***	4.72 e^{-15}***

NNum (Nodule number); NDW (nodule dry weight); Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). Values (mean ± standard deviation) with the same superscript letter within a column are not statistically different at the 5% probability according to Tukey test.

isolated from peanut. In contrast, the commercial strain USDA3187 showed no significant difference compared to the control. However, the nodule dry mass was significantly different only in plants inoculated with ISRA519 (Table 2).

In the *Essamay* cultivar, plants inoculated with the indigenous strains ISRA453, ISRA454, ISRA519 and ISRA534 showed a significant increase in nodule formation compared to the control. In addition, ORS3640, isolated from siratro plants, also showed a significant increase in the number of nodules. The reference strains LMG9283 and USDA3187 showed no significant difference in nodule formation compared to the control. Thus, the native strains appear to be more efficient in increasing the number of nodules in the *Essamay* cultivar, and no significant difference in nodule dry mass was found between treatments (Table 2).

Compared to the control plants, only inoculation with ISRA519 and ISRA534 showed a significant increase in nodule numbers when inoculated on the traditional cultivars *55-437* and *Fleur 11*, and no significant difference was found between treatments for the nodule dry mass. For the cultivar *Sunu Gaal*, inoculation with ISRA519 showed a significant increase in nodule formation (Table 2).

Estimated leaf chlorophyll content

For the six cultivars, leaf chlorophyll content at 30, 45 and 65 DAP ranged from 25.6 to 40.1 (Table S1), 30.5 to 44.2 (Table S2) and 25.5 to 39.3 (Table 3), respectively, and was higher for peanut cultivar *Amoul Morom*. The data showed no significant difference between the inoculated and non-inoculated plants, for most of the inoculated strains, irrespective of the cultivar. However, for cultivar 55-437, the leaf chlorophyll content increased significantly at 30 DAP for plants inoculated with ISRA454, ORS3640 and LMG9283 (Table S1). At 45 DAP only the plants inoculated with ISRA534 and ISRA538 showed a significant increase in leaf chlorophyll content compared to the control (Table S2). At 65 DAP, inoculation with ISRA453 and ISRA454 showed a significant increase in leaf chlorophyll content compared to the control (Table 3). In addition, the leaf

Table 3. Estimated leaf chlorophyll content at 65 days after planting in response to peanut cultivars (*A. hypogaea*) single inoculation with *Bradyrhizobium* spp. Strains.

Treatments	Peanut cultivars				
	Amoul Morom	***Essamaye***	***55-437***	***Fleur11***	***Sunu Gaal***
ISRA400	37.5±0.96^{ab}	29.3±1.85^{bc}	28.0±1.24^{ab}	28.5±3.80^{b}	32.1±2.16^{ab}
ISRA453	39.3±1.56^{a}	33.6±1.08^{a}	30.2±1.79^{a}	32.0±1.26^{ab}	35.5±2.39^{a}
ISRA454	39.3±1.93^{a}	31.9±0.88^{ab}	30.1±1.96^{a}	33.5±1.71^{a}	33.6±3.20^{ab}
ISRA519	37.6±0.81^{ab}	30.1±2.00^{abc}	27.2±3.41^{ab}	30.5±0.59^{ab}	34.3±3.28^{ab}
ISRA534	37.5±1.99^{ab}	30.8±1.34^{abc}	29.1±1.42^{ab}	29.6±1.42^{ab}	34.4±1.47^{ab}
ISRA538	35.0±2.26^{b}	31.3±1.63^{abc}	28.0±1.72^{ab}	29.4±1.82^{ab}	32.1±2.32^{ab}
ORS3640	38.1±0.67^{ab}	28.5±1.30^{bc}	27.6±1.72^{ab}	29.1±1.51^{ab}	32.2±2.24^{ab}
ORS3644	35.2±1.85^{ab}	29.8±1.77^{abc}	28.8±1.73^{ab}	29.6±1.38^{ab}	32.4±2.27^{ab}
LMG9283	37.9±1.62^{ab}	30.7±1.86^{abc}	26.6±1.67^{ab}	28.3±1.02^{b}	33.4±1.37^{ab}
USD3187	35.7±2.95^{ab}	30.7±2.34^{abc}	28.0±0.30^{ab}	30.7±2.76^{ab}	32.8±1.09^{ab}
Control	34.5±1.64^{b}	27.9±1.26^{c}	25.5±1.01^{b}	28.2±1.75^{b}	29.6±1.49^{ab}
Mea±sd	37.1±2.25	30.4±2.09	28.1±2.08	29.9±2.31	33.0±2.47
CV (%)	4.632	5.356	6.355	6.409	6.736
pValue	0.002**	0.0019**	0.021*	0.009**	0.069^{ns}

Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). Values (mean ± standard deviation) with the same superscript letter within a column are not statistically different at the 5% probability according to Tukey test.

chlorophyll content of the leaves for the control plants was lower than that of the inoculated plants for all cultivars.

Growth response of peanut cultivars to bradyrhizobial inoculation

The results showed that the traditional cultivars responded better to the bradyrhizobial inoculation than the modern cultivars, with the exception of *Sunu Gaal*, which is genetically close to *Fleur 11* (Faye I. personal communication). Of the ten treatments tested in this study, 40% showed the ability to increase plant height in the cultivar *Amoul Morom*, 20% in *Essamaye*, 80% in 55-437, 20% in Fleur 11, and 50% in *Sunu Gaal*. Inoculation with 30 and 20% of our collection showed the ability to improve biomass production in the traditional cultivars 55-437 and Fleur 11, respectively. However, none of the bradyrhizobial strains improved this growth parameter in the modern cultivars *Amoul Morom*, *Essamay* and *Sunu Gaal*.

For cultivar *55-437*, only the reference strain LMG9283 and the indigenous strain ISRA400 showed no significant difference compared to the control (Table 4). In contrast to the LMG9283, the ISRA400 significantly increased the plant height but only at 30 DAP (Table S3). The other inoculated strains showed a significant increase in plant height at 65 DAP. The highest plant height (43.2cm ± 4.74cm) was obtained with strain 0RS3644, isolated from siratro. Four indigenous strains (ISRA400, ISRA453, ISRA454 and ISRA534) showed a significant increase in plant height for the *Amoul Morom* cultivar at both 45 and 65 DAP (Table 4 and Table S4). Only the indigenous strain ISRA453 showed a significant increase for the cultivar *Essamay* at 65 DAP. Interestingly, this strain also showed a significant increase in plant height for the cultivars *Fleur 11* (at 65 DAP) and *Sunu Gaal* (at 45 and 65 DAP). Only the indigenous strains ISRA453, ISRA519, ISRA534 and ISRA538 isolated from cultivar Fleur 11 showed a significant increase in plant height with cultivar *Sunu Gaal*. However, none of the inoculated strains showed a significant difference in collar diameter for all cultivars (Table 4). Based on these results, it can be assumed that the peanut cultivar *55-437* responded better to the bradyrhizobial inoculation in terms of growth.

Peanut dry matter and yield attributes

Both shoot and root dry biomass showed no significant difference between treatments for the modern peanut cultivars *Amoul Morom*, *Essamay* and *Sunu Gaal*, but were significantly higher for the traditional cultivars *55-437* and *Fleur 11* when inoculated with ORS3644 and LMG9283 for the former and ISRA453 and ISRA454 for the latter (Table 5).

Yield characteristics were improved in 20% of the treatments for the cultivar *Essamay* and in 10% of the treatments for the cultivars *55-437*, *Fleur 11* and *Amoul Morom*. As the plants were harvested before maturity, it is expected that the yield at pod maturity of the inoculated plants will be significantly higher. In terms of pod yield attributes, the inoculated strains ISRA453, ISRA454,

Table 4. Growth (plant height and collar diameter) response of peanut cultivars (*A. hypogaea*) to single inoculation with *Bradyrhizobium* spp. 65 days after planting.

Treatments	Peanut cultivars									
	Amoul Morom		***Essamaye***		***55-437***		***Fleur11***		***Sunu Gaal***	
	Height (cm)	**CD (mm)**	**Height (cm)**	**CD (mm)**	**Height (cm)**	**CD (mm)**	**Height (cm)**	**CD (mm)**	**Height (cm)**	**CD (mm)**
ISRA400	29.3±2.56^{a}	7.70±0.67^{a}	30.2±1.11^{c}	6.17±0.56^{ab}	33.8±2.11^{de}	5.65±0.27^{ab}	30.3±2.12^{d}	5.62±0.36^{ab}	31.4±1.68^{d}	5.03±0.53^{a}
ISRA453	28.1±1.48^{ab}	6.20±0.76^{b}	35.2±1.17^{a}	4.93±0.41^{bc}	40.8±4.75^{abc}	5.73±0.48^{ab}	36.5±1.96^{a}	5.03±0.37^{ab}	36.7±3.49^{abc}	5.02±0.55^{a}
ISRA454	27.8±0.91^{ab}	7.30±0.38^{ab}	30.6±0.48^{bc}	5.91±0.58^{ab}	37.7±0.79^{abcd}	5.70±0.54^{ab}	36.1±0.70^{ab}	5.08±0.43^{ab}	32.9±1.55^{cd}	5.99±0.39^{a}
ISRA519	27.2±0.84^{abc}	7.16±0.08^{ab}	26.5±0.71^{d}	5.27±0.17^{abc}	36.4±4.15^{bcd}	5.39±0.17^{ab}	30.5±2.40^{d}	4.84±0.56^{ab}	38.5±1.74^{ab}	4.96±0.56^{a}
ISRA534	28.1±1.01^{ab}	6.77±0.42^{ab}	33.0±3.29^{ab}	5.23±0.18^{abc}	42.9±1.55^{ab}	5.09±0.44^{ab}	34.1±0.77^{abcd}	4.74±0.40^{b}	36.9±2.46^{abc}	5.22±0.18^{a}
ISRA538	26.3±1.26^{bc}	7.07±0.05^{ab}	30.7±6.09^{c}	4.60±1.04^{c}	40.1±2.39^{abcd}	4.76±0.32^{b}	32.6±0.97^{abcd}	5.12±0.38^{ab}	40.6±0.49^{a}	4.94±0.37^{a}
ORS3640	26.1±0.48^{bc}	6.64±0.41^{ab}	29.9±1.66^{c}	6.47±0.52^{a}	38.6±1.38^{abcd}	5.42±0.44^{ab}	35.2±1.14^{abc}	6.02±1.13^{a}	34.2±0.68^{bcd}	5.27±0.47^{a}
ORS3644	27.4±0.87^{abc}	7.77±0.83^{a}	35.8±1.97^{a}	5.35±0.21^{abc}	43.2±4.74^{a}	5.32±0.30^{ab}	35.5±1.08^{abc}	5.14±0.31^{ab}	36.6±0.63^{abc}	4.89±0.64^{a}
LMG9283	25.9±1.80^{bc}	7.09±0.69^{ab}	31.9±0.84^{bc}	5.81±0.65^{abc}	35.7±0.81^{cd}	5.94±0.31^{a}	32.0±2.04^{bcd}	5.33±0.54^{ab}	31.6±1.49^{d}	5.45±0.24^{a}
USD3187	25.2±1.44^{bc}	7.84±0.65^{a}	30.7±0.46^{bc}	5.71± .26^{abc}	33.5±1.29^{de}	5.28±0.22^{ab}	34.1±2.81^{abcd}	4.88±0.46^{ab}	31.1±2.53^{d}	5.23±0.64^{a}
Control	24.5±0.44^{c}	6.75±0.67^{ab}	31.5±1.35^{bc}	6.28 ±0.41^{a}	27.7±1.33^{e}	5.37±0.84^{ab}	31.6±2.06^{cd}	5.52±0.28^{ab}	30.9±1.63^{d}	5.08±0.86^{a}
Mea±sd	26.9±1.80	7.11±0.70	31.5±3.21	5.61±0.73	37.3±5.03	5.42±0.49	33.5±2.64	5.21±0.59	34.5±3.56	5.19±0.56
CV (%)	4.954	8.002	7.439	9.205	7.294	7.958	5.316	10.058	5.522	10.233
pValue	0.0007***	0.008**	0.0003***	0.0002***	4.11 e^{-08}***	0.035*	3.05 e^{-05}***	0.050*	5.13 e^{-08}***	0.231^{ns}

CD (Collar Diametter); Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). Values (mean ± standard deviation) with the same superscript letter within a column are not statistically different at the 5% probability according to Tukey test.

ISRA519, ISRA538 and LMG9283 showed a better agronomic performance for the cultivars *55-437*, *Fleur 11*, *Essamaye* and *Amoul Morom*, respectively. Some of the other strains showed higher pod number and pod dry mass than the control, but these were not statistically significant (Table 6). Cultivar *Fleur 11* had yielded more than the other four cultivars with a maximum of 1.90 ± 0.78 g plant^{-1} in the ISRA519 treatment.

DISCUSSION

Biofertilizers play an important role in increasing peanut (*A. hypogaea* L.) yield by scavenging atmospheric nitrogen (Chotangui et al., 2022), increasing phosphorus availability (Xiang et al., 2022) or by promoting growth (Frezarin et al., 2023). The aim of this study was to isolate the most specific and effective *Bradyrhizobium* spp. strain for five peanut cultivars and to determine the degree of variability in the cultivar response toward inoculation. It was observed that the strains tested in the present study promoted an increase in various parameters analyzed. Specifically, indigenous *Bradyrhizobium* isolates were more effective in improving the growth, leaf chlorophyll content and yield parameters compared to the reference strains, and the effectiveness of the inoculated strains depended on the peanut genotype used.

Peanut is endowed with nitrogen-fixing ability and can form nodules if it finds compatible soil rhizobia. In general, nodule formation depends on the number of infective rhizobia available at the root infection sites. The more infective rhizobia there is, the greater the number of nodules, although nodulation is also governed by both bacteria and intrinsic plant factors. In this study, five peanut cultivars were individually inoculated with 5 mL of a rhizobial suspension containing 10^8 cells mL^{-1}. Although the soil substrate used contains a rhizobial population greater than 10^3 cells g^{-1} (Sene et al., 2010), it was expected that the peanut cultivars would respond positively to rhizobial inoculation in terms of nodulation. The results showed that there was a significant difference in nodule number and nodule biomass between the inoculated and uninoculated treatments for all cultivars. However, irrespective of the peanut cultivar, plants in the

Table 5. Biomass production (above-ground and root biomass) of peanut cultivars at harvest.

Treatments	Peanut cultivars									
	Amoul Morom		***Essamaye***		***55-437***		***Fleur11***		***Sunu Gaal***	
	SDW (g)	**RDW (g)**	**SDW (g)**	**RDW (g)**	**SDW (g)**	**RDW (g)**	**SDW (g)**	**RDW (g)**	**SDW (g)**	**RDW (g)**
ISRA400	4.02±0.36^{a}	1.07±0.01^{a}	3.15±0.68^{a}	1.14±0.07^{ab}	2.55±0.58^{ab}	0.72±0.21^{ab}	3.19±0.39^{abc}	0.77±0.10^{a}	3.45±0.26^{a}	0.67±0.19^{a}
ISRA453	3.98±0.14^{a}	1.06±0.02^{a}	3.23±1.04^{a}	0.63±0.06^{c}	2.63±0.51^{ab}	0.86±0.14^{ab}	3.70±0.43^{ab}	0.77±0.07^{a}	3.91±0.39^{a}	0.72±0.25^{a}
ISRA454	4.06±0.43^{a}	1.11±0.04^{a}	4.02±0.35^{a}	0.92±0.19^{abc}	3.01±0.51^{ab}	0.94±0.11^{ab}	3.94±0.31^{a}	0.71±0.11^{a}	2.44±0.23^{a}	0.60±0.16^{a}
ISRA519	4.53±0.64^{a}	1.25±0.22^{a}	2.66±0.39^{a}	0.75±0.19^{bc}	2.50±0.37^{ab}	0.72±0.06^{ab}	2.82±0.06^{bc}	0.65±0.19^{a}	3.18±0.39^{a}	0.52±0.08^{a}
ISRA534	3.71±0.72^{a}	1.18±0.33^{a}	3.53±1.08^{a}	0.80±0.10^{bc}	3.00±0.30^{ab}	0.80±0.14^{ab}	3.64±0.21^{abc}	0.74±0.10^{a}	3.36±0.76^{a}	0.54±0.05^{a}
ISRA538	3.98±0.58^{a}	1.00±0.08^{a}	2.65±0.24^{a}	0.73±0.14^{c}	2.52±0.78^{ab}	0.63±0.12^{ab}	2.86±0.40^{abc}	0.69±0.09^{a}	3.89±0.89^{a}	0.63±0.07^{a}
ORS3640	3.97±0.88^{a}	1.18±0.10^{a}	3.18±0.65^{a}	0.91±0.27^{abc}	2.96±0.60^{ab}	0.97±0.21^{a}	3.63±0.67^{abc}	0.69±0.20^{a}	3.74±0.87^{a}	0.62±0.17^{a}
ORS3644	3.92±0.17^{a}	1.22±0.28^{a}	3.03±0.56^{a}	0.85±0.12^{abc}	3.27±0.86^{a}	0.78±0.19^{ab}	3.62±0.29^{abc}	0.85±0.13^{a}	3.41±0.90^{a}	0.64±0.01^{a}
LMG9283	3.53±0.39^{a}	1.02±0.03^{a}	3.08±0.71^{a}	0.91±0.09^{abc}	3.85±0.19^{a}	0.78±0.16^{ab}	2.83±0.46^{abc}	0.89±0.07^{a}	3.15±1.05^{a}	0.56±0.08^{a}
USD3187	3.97±0.20^{a}	1.10±0.08^{a}	3.85±0.19^{a}	1.22±0.19^{a}	2.69±1.23^{ab}	0.91±0.14^{ab}	3.38±0.86^{abc}	0.60±0.02^{a}	2.44±0.30^{a}	0.80±0.10^{a}
Control	3.36±0.70^{a}	0.90 ± 0.11^{a}	2.73±0.25^{a}	0.94±0.22^{abc}	1.45±0.47^{b}	0.60±0.10^{b}	2.56±0.55^{c}	0.75±0.11^{a}	2.91±1.24^{a}	0.72±0.07^{a}
Mea±sd	0.91±0.55	1.10±0.17	3.19±0.71	0.89±0.22	2.77±0.80	0.79±0.17	3.29±0.60	0.74±0.13	3.24±0.81	0.64±0.14
CV (%)	13.525	13.833	19.806	18.168	23.242	18.934	13.927	16.315	22.841	20.534
pValue	0.266^{ns}	0.091^{ns}	0.057^{ns}	0.0005***	0.004**	0.020*	0.001**	0.083^{ns}	0.097^{ns}	0.13^{ns}

SDW (Shoot dry weight); RDW (Root dry weight); Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ' , 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). Values (mean ± standard deviation) with the same superscript letter within a column are not statistically different at the 5% probability according to Tukey test.

Bradyrhizobium strain ISRA519 treatment were more nodulated than the other inoculated strains and the uninoculated plants, indicating that this strain had a high capacity for nodule initiation and subsequent nodule development. The result also showed that this strain was the only inoculant to have a significant effect on pooled nodule dry weight. This result is consistent with a previous report by Zaiya et al. (2018), who classified ISRA519 as the most nodulating isolate.

The data of this study support a cultivar-*Bradyrhizobium* specificity. The results showed that nodulation was more pronounced in some cultivars than in others. In particular, strain ISRA519 showed the highest nodule number and nodule dry weight with the traditional peanut cultivars *55-437* and *Fleur 11* and the modern cultivar *Sunu Gaal* (which is genetically very close to the cultivar *Fleur 11*) compared to the modern cultivars *Amoul Morom* and *Essamay*. In contrast, most of the other inoculated *Bradyrhizobium* strains showed increased nodulation with the latter cultivars rather than with the former. This confirmed the role of plant genotype in nodule formation with inoculated rhizobial strains already reported in soybean (*Glycine max*) (Meghvansi et al., 2008). This may have been the reason for the different compatibility of the tested isolates with the tested peanut cultivars. This observation was consistent with the findings of Chen et al. (2003) in Argentine soil and Argaw (2017) in Ethiopian soil that there was a cultivar-*Bradyrhizobium* strain specificity. On the other hand, the indigenous strains seemed to be more efficient in increasing the number of nodules compared to the reference strains, regardless of the cultivar used. This is particularly observed for the exotic strain USDA3187 and is probably due to the fact that the native strains are better adapted and therefore have an advantage in nodule colonization. Similarly, previous results where rhizobial inoculation had no significant effect on nodulation have been reported (Castro et al., 1999; Bogino et al., 2006, 2008; Chotangui et al., 2022) and support our data.

Peanut was considered a highly "promiscuous" species (Bogino et al., 2006), being nodulated by a wide variety of rhizobia. Thus, the response of

Table 6. Yield attributes (number of pods per plant, pod weight) of peanut cultivars at harvest.

Treatments	Peanut cultivars									
	Amoul Morom		***Essamaye***		***55-437***		***Fleur11***		***Sunu Gaal***	
	Pod number	**Weight of pods (g)**	**Pod number**	**Weight of pods (g)**	**Pod number**	**Weight of pods (g)**	**Pod number**	**Weight of pods (g)**	**Pod number**	**Weight of pods (g)**
ISRA400	7.25±0.96^{a}	0.59±0.11^{ab}	5.25±1.71^{a}	0.45±0.23^{c}	3.25±1.26^{ab}	0.76±0.49^{ab}	5.25±1.50^{a}	1.06±0.28^{b}	5.00±1.41^{ab}	0.85±0.20^{ab}
ISRA453	5.00±1.41^{ab}	0.48±0.18^{ab}	5.50±1.29^{a}	1.55±0.22^{a}	3.25±0.96^{ab}	0.88±0.21^{ab}	5.75±0.96^{a}	1.55±0.33^{ab}	7.00±1.41^{a}	0.66±0.20^{ab}
ISRA454	4.75±0.96^{ab}	0.52±0.10^{ab}	5.50±2.38^{a}	0.78±0.11^{c}	6.00±1.63^{a}	1.08±0.30^{a}	7.00±0.82^{a}	1.56±0.27^{ab}	4.25±0.96^{ab}	0.49±0.10^{b}
ISRA519	5.75±09.6^{ab}	0.81±0.25^{ab}	5.00±0.82^{a}	0.81±0.27^{c}	4.25±0.96^{ab}	0.42±0.15^{b}	7.00±1.83^{a}	1.90±0.78^{a}	6.50±1.29^{ab}	0.54±0.10^{ab}
ISRA534	4.00±1.00^{ab}	0.64±0.31^{ab}	5.75±0.96^{a}	0.74±0.07^{c}	4.75±0.96^{ab}	0.44±0.24^{ab}	7.50±1.00^{a}	1.61±0.20^{ab}	4.75±1.71^{ab}	0.61±0.06^{ab}
ISRA538	6.50±2.52^{ab}	0.90±0.22^{a}	3.50±0.58^{a}	0.50±0.14^{c}	3.50±0.58^{ab}	0.39 ± 0.18^{b}	6.50±1.29^{a}	1.30±0.20^{ab}	5.33±0.58^{ab}	0.59±0.09^{ab}
ORS3640	6.50±1.29^{ab}	0.66±0.17^{ab}	5.25±2.06^{a}	0.83±0.21^{c}	5.25±1.50^{ab}	0.87±0.39^{ab}	7.75±1.26^{a}	1.49±0.10^{ab}	6.25±1.26^{ab}	0.95±0.12^{a}
ORS3644	4.00±0.82^{b}	0.48±0.06^{ab}	5.50±0.58^{a}	0.89±0.15^{bc}	5.25±1.50^{ab}	0.89±0.19^{ab}	6.50±1.29^{a}	1.28±0.19^{ab}	6.00±2.45^{ab}	0.69±0.22^{ab}
LMG9283	5.00±0.82^{ab}	0.48±0.17^{ab}	4.25±0.50^{a}	1.35±0.30^{ab}	4.75±1.71^{ab}	0.87±0.23^{ab}	5.25±0.96^{a}	1.00±0.34^{b}	3.75±1.26^{ab}	0.78±0.36^{ab}
USD3187	6.33±0.58^{ab}	0.63±0.03^{ab}	4.50±0.58^{a}	0.90±0.22^{bc}	5.00±0.82^{ab}	0.82±0.22^{ab}	5.75±2.36^{a}	0.98±0.17^{b}	3.25±1.50^{b}	0.53±0.13^{ab}
Control	5.00±1.41^{ab}	0.41±0.26^{b}	5.50±0.58^{a}	0.72±0.23^{c}	2.75±0.96^{b}	1.03±0.04^{ab}	4.75±1.50^{a}	1.06±0.24^{b}	4.25±0.96^{ab}	0.59±0.10^{ab}
Mea±sd	5.48±1.52	0.60±0.21	5.05±1.29	0.86±0.36	4.36±1.46	0.77±0.33	6.27±1.56	1.34±0.41	5.12±1.72	0.66±0.21
CV (%)	23.403	29.791	25.118	24.012	27.925	34.656	22.460	24.608	28.105	26.672
pValue	0.018*	0.014*	0.34^{ns}	2.46 e^{-07}***	0.010*	0.005**	0.069^{ns}	0.004**	0.013*	0.017*

Significant codes:0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). Values (mean ± standard deviation) with the same superscript letter within a column are not statistically different at the 5% probability according to Tukey test.

peanut to rhizobial inoculation has always been questionable worldwide: India (Gaur et al., 1974; Nambiar, 1985; Wange, 1989; Joshi et al., 2008), Israel (Schiffmann and Alper, 1968), Brazil (Cardoso et al., 2009), Argentina (Castro et al., 1999; Bogino et al., 2006; 2008), and Cameroon (Chotangui et al., 2022). A positive response has already been observed by Sene et al. (2010) in the selected site, that is, Sangalkam, where the population size of the indigenous rhizobia is greater than 10^3 cells g^{-1}. Indeed, most of the inoculated plants of cultivars 55-437 were positively affected at 45 DAP and positive responses were also observed at 65 DAP in terms of plant height, shoot dry weight and pod number and dry matter. Consistent with its effect on nodulation, the indigenous strain ISRA519 significantly affected plant growth of cultivars *55-437* and *Sunu Gaal* and pod yield parameters of cultivar *Fleur 11*, but no significant increase was observed for the remaining modern cultivars. This confirms the cultivar-*Bradyrhizobium* specificity found for nodulation and supported by several authors (Chen et al., 2003; Meghvansi et al., 2008; Argaw, 2017). Although the precise drivers of the variation in root nodulation rates and host plant response among different crop genotypes remain poorly understood, it has been suggested, for instance, that modern crop breeding may have negatively affected the ability to establish microbial symbioses (Martín-Robles et al., 2018, 2020; Sawers et al., 2018). As breeding programs generally aim to maximize crop yield in high-input production systems, it is likely that a breeding process accompanied by high fertilization rates may select crop genotypes that are less responsive to the root microbial symbioses, as has been suggested by Parvin et al. (2021) with arbuscular mycorrhizal fungi and rice cultivars.

Although the inoculated plants performed better than the non-inoculated plants, the expected improvements were not achieved for some inoculated strains as most of the isolates were previously selected for their efficacy on peanut and other legume species (Sene et al., 2010, 2012, 2013; Zaiya et al., 2018). The results showed that most of the inoculated strains that have induced improvements in plant growth and

yield parameters were from the indigenous collection of bradyrhizobia. Inoculation with ISRA453 and ISRA454 showed the better to increase plant growth and biomass production in all cultivars. The performance of the indigenous bradyrhizobia was demonstrated in the work of Zaiya et al. (2018) and confirmed in the present study. This is reflected in the leaf chlorophyll content shown in the SPAD readings, which indicates improved nitrogen fixation of these inoculated strains compared to the reference strain USDA3187 formulated for peanut cultivation. Indeed, chlorophyll pigment is an indicator of the level of nitrogen assimilation and is responsible for the green color of the leaves (Deroche, 1983). The efficiency of nitrogen fixation in peanut was previously reported to result in the accumulation of nitrogen in plants which in turn reflected the synthesis of chlorophyll (Nageswara et al., 2001). The failure of inoculation with the *Bradyrhizobium* strain USDA3187 to elicit a response in peanut cultivars could be attributed to the presence of highly competitive but ineffective indigenous strains that exclude the inoculated strains from occupying the nodules, as has been suggested in previous work by several authors (Castro et al., 1999; Bogino et al., 2006, 2008; Sene et al., 2010). In contrast, *Bradyrhizobium* spp. LMG9283 has promoted an increase in plant height and shoot dry weight of peanut cultivar *55-437* and improved yield parameters of cultivar *Essamay*. The strain LMG9283 was isolated in the Senegalese peanut basin and has already been recognized for its agronomic performance on peanut (Dhery and Dreyfus, 1991; Sene et al., 2010). Thus, the indigenous bradyrhizobia seem to be more efficient in increasing nodulation, plant growth and yield parameters.

Conclusion

The demand for microbial inoculants is increasing, driven by the need for sustainable and environmentally friendly agricultural practices and safer and healthier food. In order to select the best *Bradyrhizobium* spp. inoculants for Senegalese peanut cultivars, it was hypothesized that the response of peanut toward rhizobial inoculation is cultivar dependent and that there is a different degree of variability between traditional and modern cultivars. The results of this study showed that the strains tested promoted increases in various parameters analyzed. Specifically, indigenous *Bradyrhizobium* isolates were more effective in improving the growth, leaf chlorophyll content and yield parameters compared to the reference strains. This suggests that inoculation of peanut cultivars with exotic inoculants to improve plant growth and yield parameters is not necessary in the study soil. According to this study, it would be useful to promote the use of indigenous strains that perform well with the peanut cultivars studied. We also showed that the efficacy of the inoculated strains depended on the peanut genotype used and the result highlighted the need for cultivar-specific selection of *Bradyrhizobium* to reap the benefits of nitrogen fixation and improve inoculation success in peanut. In addition, traditional peanut cultivars such as *55-437* responded better than the modern cultivars, demonstrating the differential feedback between peanut cultivars and *Bradyrhizobium* spp. partners. As the response of peanut to rhizobial inoculation has been questionable in West Africa, the increase in yield of the tested peanut cultivars should be considered as new promising data for the adoption of rhizobium technology for peanut improvement. Yet, this cannot be considered as a success of inoculation of peanut with rhizobial strains and field inoculation over several years is required.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY DATA

Table S1. Leaf chlorophyll content at 30 days after planting in response to peanut cultivars inoculation with *Bradyrhizobium* sp. Strains.

Treatments	Peanut cultivars				
	Amoul morom	***Essamaay***	***55-437***	***Fleur 11***	***Sunu Gaal***
ISRA400	38.1 ± 1.06^{a}	33.0 ± 2.39^{a}	31.0 ± 3.37^{a}	33.3 ± 1.69^{abc}	30.2 ± 2.98^{a}
ISRA453	39.0 ± 3.16^{a}	31.0 ± 1.04^{a}	28.0 ± 0.95^{ab}	30.3 ± 1.02^{bc}	30.2 ± 3.29^{a}
ISRA454	39.0 ± 0.91^{a}	35.0 ± 4.42^{a}	31.9 ± 1.40^{a}	33.7 ± 2.47^{ab}	32.7 ± 0.78^{a}
ISRA519	37.1 ± 3.72^{a}	29.8 ± 1.51^{a}	27.9 ± 1.30^{ab}	31.4 ± 1.19^{abc}	30.8 ± 1.84^{a}
ISRA534	36.3 ± 0.70^{a}	31.0 ± 2.21^{a}	30.5 ± 1.60^{ab}	29.1 ± 0.61^{c}	32.8 ± 4.16^{a}
ISRA538	39.2 ± 0.70^{a}	31.3 ± 1.20^{a}	28.6 ± 2.40^{ab}	31.8 ± 1.23^{abc}	32.1 ± 2.72^{a}
ORS3640	37.0 ± 1.26^{a}	30.6 ± 1.05^{a}	31.0 ± 1.16^{a}	35.6 ± 3.61^{a}	33.2 ± 2.09^{a}
ORS3644	40.1 ± 2.73^{a}	32.8 ± 1.13^{a}	30.2 ± 1.54^{ab}	29.4 ± 1.36^{bc}	33.2 ± 0.48^{a}
LMG9283	37.6 ± 2.87^{a}	34.1 ± 2.59^{a}	31.3 ± 0.41^{a}	31.7 ± 2.21^{abc}	32.0 ± 3.52^{a}
USD3187	38.2 ± 1.83^{a}	34.5 ± 4.94^{a}	29.6 ± 1.20^{ab}	31.1 ± 1.68^{bc}	33.1 ± 2.66^{a}
Control	35.9 ± 1.47^{a}	29.9 ± 2.31^{a}	25.6 ± 4.15^{b}	28.9 ± 0.81^{c}	29.7 ± 1.80^{a}
Mea ± sd	38.0 ± 2.26	32.1 ± 2.90	29.6 ± 2.56	31.5 ± 2.56	31.8 ± 2.63
CV (%)	5.689	8.068	6.971	5.795	8.234
pValue	0.23^{ns}	0.0549^{ns}	0.000144 ***	3.05 e^{-05} ***	0.437^{ns}

Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). In columns, means with identical superscript letters are statistically equivalent at the 5% probability level.

Table S2. Leaf chlorophyll content at 45 days after planting in response to peanut cultivars inoculation with *Bradyrhizobium* sp. Strains.

Treatments	Peanut cultivars				
	Amoul morom	***Essamaay***	***55-437***	***Fleur 11***	***Sunu Gaal***
ISRA400	39.1 ± 1.54^{abc}	34.5 ± 1.56^{a}	32.4 ± 2.03^{ab}	32.6 ± 0.41^{a}	34.2 ± 2.44^{a}
ISRA453	42.0 ± 1.68^{ab}	35.9 ± 2.58^{a}	32.1 ± 1.72^{ab}	35.4 ± 2.34^{a}	38.5 ± 1.76^{a}
ISRA454	39.3 ± 2.79^{abc}	34.0 ± 1.91^{a}	32.0 ± 0.57^{ab}	33.6 ± 1.73^{a}	34.3 ± 2.85^{a}
ISRA519	41.7 ± 1.84^{abc}	35.1 ± 0.54^{a}	33.4 ± 3.68^{ab}	35.3 ± 2.50^{a}	35.9 ± 4.09^{a}
ISRA534	41.4 ± 0.85^{abc}	37.2 ± 3.12^{a}	36.8 ± 2.21^{a}	33.0 ± 1.25^{a}	38.7 ± 1.08^{a}
ISRA538	44.2 ± 4.65^{a}	36.3 ± 3.81^{a}	35.7 ± 2.33^{a}	36.2 ± 2.62^{a}	35.5 ± 3.32^{a}
ORS3640	38.5 ± 0.52^{bc}	35.2 ± 2.51^{a}	32.4 ± 1.40^{ab}	33.1 ± 1.45^{a}	34.3 ± 1.44^{a}
ORS3644	40.1 ± 1.84^{abc}	34.1 ± 1.30^{a}	33.2 ± 2.28^{ab}	32.4 ± 3.39^{a}	34.3 ± 2.62^{a}
LMG9283	36.6 ± 1.47^{c}	35,5 ± 1,79^{a}	31,9 ± 2,39^{ab}	34.3 ± 1.07^{a}	33.3 ± 4.02^{a}
USD3187	38.4 ± 2.27^{bc}	35.0 ± 1.89^{a}	32.3 ± 0.51^{ab}	34.3 ± 2.59^{a}	35.1 ± 1.46^{a}
Control	38.3 ± 0.59^{bc}	33.9 ± 1.93^{a}	30.5 ± 1.24^{b}	32,4 ± 1,45^{a}	33,0 ± 1,14^{a}
Mea ± sd	40.0 ± 2.83	35.1 ± 2.21	33.0 ± 2.49	33.9 ± 2.22	35.2 ± 2.90
CV (%)	5.398	6.408	6.202	6.097	7.332
P value	0.00148**	0.591^{ns}	0.0073**	0.134^{ns}	0.0517^{ns}

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd (mean ± standard deviation); CV (coefficient of variation). In columns, means with identical superscript letters are statistically equivalent at the 5% probability level.

Table S3. Plant height (cm) of peanut cultivars at 30 days after planting under inoculation with *Bradyrhizobium* sp. Strains.

Treatments	Peanut cultivars				
	Amoul Morom	***Essamaay***	***55-437***	***Fleur 11***	***Sunu Gaal***
ISRA400	19.2 ± 0.85^{a}	20.0 ± 2.13^{b}	28.2 ± 1.55^{abcd}	22.3 ± 1.29^{ab}	24.1 ± 2.66^{abc}
ISRA453	17.6 ± 1.50^{ab}	26.3 ± 1.33^{a}	31.3 ± 1.66^{a}	24.7 ± 2.08^{ab}	24.4 ± 1.88^{abc}
ISRA454	19.1 ± 0.99^{ab}	20.6 ± 0.45^{b}	25.9 ± 0.22^{cde}	22.0 ± 1.53^{b}	22.8 ± 1.04^{bc}
ISRA519	17.5 ± 0.71^{ab}	18.9 ± 0.48^{b}	27.0 ± 3.19^{bcd}	21.5 ± 0.71^{b}	24.3 ± 0.99^{abc}
ISRA534	17.5 ± 1.50^{ab}	21.8 ± 1.50^{ab}	29.4 ± 1.65^{abc}	26.4 ± 1.63^{a}	22.1 ± 2.66^{bc}
ISRA538	18.0 ± 1.08^{ab}	18.9 ± 1.15^{b}	28.4 ± 1.38^{abcd}	21.9 ± 1.84^{b}	26.8 ± 2.36^{ab}
ORS3640	17.3 ± 0.71^{ab}	21.6 ± 0.52^{ab}	28.5 ± 0.61^{abcd}	24.2 ± 2.69^{ab}	27.8 ± 0.51^{a}
ORS3644	17.4 ± 0.83^{ab}	22.8 ± 0.65^{ab}	30.1 ± 1.53^{ab}	22.4 ± 0.34^{ab}	24.3 ± 1.03^{abc}
LMG9283	17.7 ± 0.81^{ab}	22.6 ± 4.55^{ab}	24.9 ± 1.44^{de}	21.4 ± 1.92^{b}	22.3 ± 2.11^{bc}
USD3187	16.5 ± 0.00^{b}	21.6 ± 2.24^{ab}	28.6 ± 1.10^{abcd}	22.1 ± 0.97^{b}	21.9 ± 1.4 ^{c}
Control	17.7 ± 1.09^{ab}	23.0 ± 2.42^{ab}	22.3 ± 1.41^{e}	21.3 ± 2.43^{b}	20.1 ± 1.65^{c}
Mea ± sd	17.8 ± 1.13	21.6 ± 2.67	27.7 ± 2.81	22.7 ± 2.18	23.6 ± 2.62
CV (%)	5.574	9.101	5.759	7.587	7.554
P value	0.0446*	0.000385***	1.76e^{-07}***	0.00258**	5.8e^{-05}***

Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test) ; ns (not significant difference) ; Mea ± sd (mean ± standard deviation) ; CV (coefficient of variation). In columns, means with identical superscript letters are statistically equivalent at the 5% probability level.

Table S4. Plant height (cm) of peanut cultivars at 45 days after planting under inoculation with *Bradyrhizobium* sp. Strains.

Treatments	Peanut cultivars				
	Amoul Morom	***Essamaay***	***55-437***	***Fleur 11***	***Sunu Gaal***
ISRA400	24.4 ± 0.63^{a}	26.0 ± 1.83^{bc}	32.5 ± 2.48^{abc}	26.8 ± 1.34^{c}	28.8 ± 1.95^{bcd}
ISRA453	24.8 ± 3.62^{a}	31.9 ± 1.72^{a}	38.8 ± 4.73^{a}	33.1 ± 0.95^{a}	32.9 ± 3.30^{ab}
ISRA454	24.6 ± 0.99^{a}	27.0 ± 1.15^{abc}	32.2 ± 1.39^{abc}	29.7 ± 1.57^{abc}	27.8 ± 1.55^{cd}
ISRA519	24.3 ± 1.19^{ab}	23.9 ± 0.85^{c}	33.2 ± 3.33^{ab}	27.4 ± 2.63^{bc}	32.9 ± 1.89^{ab}
ISRA534	23.6 ± 1.05^{ab}	28.9 ± 2.83^{abc}	37.5 ± 1.56^{a}	31.9 ± 1.25^{ab}	30.7 ± 2.96^{bcd}
ISRA538	23.7 ± 2.08^{ab}	27.9 ± 3.54^{abc}	34.9 ± 3.49^{ab}	29.5 ± 1.87^{abc}	36.0 ± 0.50^{a}
ORS3640	21.3 ± 0.85^{ab}	27.9 ± 1.21^{abc}	34.1 ± 1.38^{ab}	31.4 ± 2.50^{abc}	32.5 ± 1.68^{abc}
ORS3644	22.8 ± 0.33^{ab}	30.0 ± 2.94^{ab}	36.9 ± 4.23^{ab}	29.6 ± 1.91^{abc}	31.0 ± 2.00^{abcd}
LMG9283	21.9 ± 1.73^{ab}	28.1 ± 2.10^{abc}	30.5 ± 0.41^{bc}	28.5 ± 2.35^{abc}	27.9 ± 1.62^{cd}
USD3187	21.7 ± 1.08^{ab}	27.5 ± 2.65^{abc}	34.5 ± 2.13^{ab}	28.3 ± 3.06^{abc}	27.3 ± 1.43^{d}
Control	20.4 ± 0.74^{b}	25.1 ± 1.23^{bc}	26.4 ± 0.77^{c}	27.1 ± 1.84^{bc}	27.7 ± 1.08^{bcd}
Mea ± sd	23.1 ± 2.01	27.7 ± 2.87	33.8 ± 4.11	29.4 ± 2.66	30.4 ± 3.16
CV (%)	6.896	7.859	8.056	6.903	6.555
P value	0.00367**	0.000827***	1.86e^{-05}***	0.00105**	6e-^{06} ***

Significant codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1 (significant differences according to Tukey test); ns (not significant difference); Mea ± sd probability level. (mean ± standard deviation); CV (coefficient of variation). In columns, means with identical superscript letters are statistically equivalent at the 5%

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Preferential expression of somatic embryogenesis in five elite genotypes of *Theobroma cacao* (L.) associated with explant type and protocols used

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This study aims to identify the suitable subculturing time, culture media (protocols) and explants type suitable for somatic embryogenesis expression in five elite genotypes of *Theobroma cacao* L. Thus, petals and staminodes explants extracted from immature flower buds of genotypes (C1, C8, C9, C14, and C16) are first grown in different media (CIM and CDM) to assess the subculturing time. Then, successive transfers in EDM medium at every 4 weeks intervals until 24 weeks. To assess culture media preference, these genotypes were grown in four different protocols (I, II, III, and IV) for callus induction. They were then successively subcultured (four times at 4 weeks intervals) into EDM. About the subculture time, embryo expression is earlier in C1, C9, C14, and C16 genotypes (2nd and 4th weeks) than C8 genotype (12 and 16th weeks). However, C1, C14, and C16 (with staminodes explants) prefer protocol IV, while their petal explants prefer protocol II. Also, C9 staminodes explants prefer protocol III while the C9 and C8 petals explants prefer protocol I. This study has identified suitable explants and protocols for somatic embryo production in the tested genotypes. Moreover, this work has laid out a new strategy to overcome recalcitrance to the somatic embryogenesis of *T. cacao* genotypes.

Key words: Explants, genotypes, somatic embryogenesis, subculture time, *Theobroma cacao* L.

INTRODUCTION

Theobroma cacao L is a cross-pollinating tree, native to the American tropics, which significantly contributes to the economies of many developing countries on the Asian, African, and Latin American continents (Myeki et al., 2022). Cocoa beans are of particular interest to the global chocolate industry (Ackah and Dompey, 2021; Perez et al., 2021). Chocolate, recognized as a healthy and nutritious food, is mainly considered medicine for

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heart diseases (Decroix et al., 2018; Meier et al., 2017; Correia et al., 2016). Due to the continuously increasing global demand for cocoa, the sustainable intensification of its production in West Africa is considered crucial (Kongor et al., 2018).

Côte d'Ivoire is the world's leading producer and exporter of cocoa beans, with a global market share of around 41%. It is expected to produce 2.2 million tons in 2021/2022, compared with 2.248 million tons in 2020/2021. However, climate change, pest infestation, soil degradation and aging plantations are the main factors contributing to the decline in production. Also, these orchards, which are mostly degraded, require replanting (Kouame et al., 2016). As a result, the country needs strategies to maintain its production and its leading position.

Furthermore, the International Agroforestry Research Center, as part of the Vision for Change (V4C) project in 2010 initiated a program whose aim was to revitalize the cocoa sector throughout Côte d'Ivoire. This has resulted in well-trained producers, higher yields, better quality production and a profitable cocoa economy that benefits the whole community. During this program, elite cocoa genotype plants were selected (Jane et al., 2016; Kouamé, 2015). The performance of these genotypes grafted onto mature plants in the field has resulted in yields exceeding 2 t/ha in the fourth year of production, and some of these genotypes show tolerance to the cocoa swollen shoot disease. In addition, the quality of cocoa beans has been determined by many factors, such as size, number, color, degree of bitterness, acidity and flavor (Kouame et al., 2016). Except that the propagation of these genotypes by the classical method is difficult to achieve due to the heterogeneity of the seeds. In fact, cocoa has various propagation systems, such as seeds, grafting, and cuttings that provide planting material for plantations (Batista et al., 2015). However, these systems are difficult to implement due to the heterogeneity of seeds, the poor architecture of plants obtained after cuttings, and the need for large quantities of grafted plants with the desired genetic and sanitary quality (Garcia et al., 2016; Batista et al., 2015). Therefore, the development of rapid and highly efficient systems to facilitate the vegetative propagation of cocoa is considered necessary. Therefore, the somatic embryogenesis approach offers an alternative strategy to produce elite planting material (in quality and quantity) for sustainable cocoa production (Isah, 2019).

Somatic embryogenesis is one of the methods of cocoa propagation with multipurpose potential ranging from genetic improvement to mass production of elite plants and germplasm conservation (Garcia et al., 2016). In *T. cacao*, several somatic embryogenesis protocols using the mineral solution, Driver and Kuniyaki (DKW, 1984), added with different growth regulators have been developed. According to Kouassi et al. (2017), the effect of combining dichlorophenoxyacetic acid (2,4-D) with kinetin is more embryogenic than with thidiazuron (TDZ). Also, reports of Li et al. (1998) showed that a concentration of 22.7 nM of TDZ combined with 2,4-D was found to be optimal for the efficient induction of somatic embryos of 19 cocoa genotypes. These protocols aimed to define an ideal medium for the optimization of somatic embryo induction. However, variability (recalcitrance and low somatic embryo expression) in genotype response is reported. Genotypes have variability in response to the protocols (Garcia et al., 2016). In previous work, research teams (Kouassi et al., 2018; Kahia et al., 2017; Garcia et al., 2016; Li et al., 1998) had conducted investigations with a specific protocol to question somatic embryogenic expression in *T. cacao* genotypes. This approach (use of a specific somatic embryogenesis protocol applied to different genotypes from a specific experimental area) resulted in specific genotypes and protocols used. To overcome the recalcitrance constraints and improve the embryogenic response of *T. cacao* genotypes, an original approach would be to test different somatic embryogenesis protocols on a set of genotypes.

Various protocols for somatic embryogenesis of *T. cacao* are in use in laboratories (Kouassi et al., 2018; Kahia et al., 2017; Li et al. 1998). The main point of divergence of these different protocols is the constitution of the culture medium at the induction stage (of somatic embryogenesis). This phase corresponds to the state of dedifferentiation or disorganization of the current genetic program and the establishment of a new genetic program expression should lead to the formation of somatic embryos.

In Côte d'Ivoire, genotypes (C1, C8, C9, C14, and C16) of *T. cacao* with good agronomic performance in the field had been selected (Kahia et al., 2017). These genotypes were previously studied and showed that C1 and C14 are the most embryogenic, and C8 is recalcitrant with a culture medium using 2,4-dichloro phenoxy acetic acid (2,4-D) and kinetin (KIN) as growth regulators (Kouassi et al., 2018). Furthermore, our research team (Kahia et al., 2017) had developed culture media to induce somatic embryos at very high rates in C9, C1, and C14 genotypes. Also, a recent study according to Henao Ramírez et al. (2018) showed a different capacity of response to genotype induction depending on the subculture time. Considering subculture time, which is a period of tissue cellular incubation in the culture medium, these authors showed on one hand that the time of subculture as usual use proves that certain treatments (hormone, medium of culture) were better than the other. On the other hand of subculture time of the tissues cellular of genotypes was limited at a specific period that may be conducive or non-conducive to somatic embryogenesis. All these approaches show different embryogenic genotypic responses. But no combined study of the protocols has yet been conducted to improve the response of *T. cacao* genotypes to somatic

Table 1. Origin and field performance of five elite cacao genotypes.

Genotype	Origin	Performance (ton/ha) (2011-2015)
C1	Côte d'Ivoire	2.3
C16	Côte d'Ivoire	4
C14	Côte d'Ivoire	2.8
C9	Trinidad	2,3
C8	Trinidad	1,8

Data of vision for change project.
Source: Authors

embryogenesis.

This work will examine the performance of somatic embryogenesis expression according to explants of a pool of *T. cacao* genotypes (C1, C8, C9, C14, and C16) in a pool of protocols and in subculture time (while calli were transferred in EDM medium at every 4 weeks intervals until 24 weeks instead of the 6 weeks usually used), in order to identify which protocol or subculture time is best suited to which type of explant of a genotype.

MATERIALS AND METHODS

Source genotypes for explants

The explants in this study consisted of staminodes and petals explants of immature flower buds of five elite cocoa genotypes with codes C1, C8, C9, C14, and C16. These genotypes are obtained by grafting and kept in the genomic bank in the experimental field of the International Research Centre for Agroforestry in Adiopodoume (Côte d'Ivoire). In this experimental field, the average annual rainfall is 1320 mm. The temperature varies from 22 to 35°C. The origin and field performance of these genotypes are presented in Table 1.

Experimental design

The experiment conducted from January to May 2021, consisted of evaluating the protocol(s) and subculture time best suited for the expression of somatic embryos of five genotypes (C1, C8, C9, C14, and C16) of *T. cacao*.

Staminodes and petals explants of the flower buds (after surface sterilization) of five genotypes were simultaneously seeded on induction media (of variable composition between the four protocols tested) for 14 and 28 days, respectively for protocols I and II, III, and IV. At the end of the 14 and 28 days in the induction media, the explants were either:

(1) subcultured in the maintenance medium (for 14 days) followed by 6 subcultures at 28-day intervals in the somatic embryo expression medium, the protocol I (Table 2);
(2) subcultured 4 times at 28-day intervals in the somatic embryo expression medium (protocols II, III, IV) (Table 2).

Culture medium

Protocol I

Three culture media were used, CIM_1 (primary callus induction medium), CDM (secondary callus development medium), and EDM (somatic embryo development medium). CIM_1 medium was prepared by mixing 100 ml of macroelements and 10 ml of microelements from Driver and Kuniyaki Walnut (DKW) with 1 ml of vitamin (100 mg/ml myoinositol + 2 mg/ml thiamine-HCL + 1 mg/ml nicotinic acid + 2 mg/ml glycine) from DKW, 20 g/l glucose, 250 mg/l glutamine, 100 mg/l myoinositol, 2 mg/l 2,4-Dichlorophenoxyacetic acid, 0.00 mg/l thidiazuron and 2g/l phytagel. The pH of the mixture was adjusted to 5.8.

The CDM medium used consists of 2.3 g of McCown's, 1 ml of Gamborg's vitamin solution (100 mg/ml myoinositol + 10 mg/ml thiamine-HCL + 1 mg/ml nicotinic acid + 1 mg/ml pyridoxine), glucose (20 g/l), 2,4-D (2 mg/l), 6-benzyl aminopurine (0.005 mg/l), and 2.2 g/l phytagel. The pH of the mixture was adjusted to 5.7.

EDM_1 medium was obtained by mixing 100 ml of macroelements, 10 ml of microelements, 1 ml of DKW Vitamin supplemented with 30 g/l sucrose, and 2 g/l phytagels. The pH of the mixture was adjusted to 5.8 (Table 2).

Protocol II

Two types of culture media were used: the callus induction medium (CIM_2) and EDM (somatic embryo development medium). The CIM_2 consisted of DKW macroelements (100 ml/l), DKW microelements (10 ml/l), DKW vitamin (1 ml), Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (4.5 µM), Kinetine (1.125 µM), Glucose (30 g/l), and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that of Protocol I (Table 2).

Protocol III

As in protocol II, two types of culture media were used: the callus induction medium (CIM_3) and the somatic embryo expression medium. The CIM_3 is made up of Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetine (2.5 µM), Glucose (30 g/l) and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that of Protocol I (Table 2).

Protocol IV

In this protocol, two types of culture media were also used: the callus induction medium (CIM_4) and the somatic embryo expression medium. The CIM_4 consists of Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetine (2.5 µM), Sucrose (34.2 g/l) and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that used in Protocol I

Table 2. Culture phases and protocols.

Phase	Biological processes	Types of culture media	Constitution of culture media			
			Protocol I (control)	Protocol II (Kouassi et al., 2017)	Protocol III (Kahia et al., 2017)	Protocol IV (experimental)
Induction (dedifferentiation)	Disorganization of the current genetic program and establishment of a new genetic program	Primary callus induction medium	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (2 mg/l), TDZ (0,005 mg/l), Glucose (20 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (4.5 µM), Kinetin (1,125 µM), Glucose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetin (2.5 µM), Glucose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20µM), Kinetin (2.5 µM), Saccharose (34,2 g/l), Phytagel (2 g/l)
Maintenance	Maintenance of the new genetic program	Secondary callus induction medium	McCown's (2.3 g/l), Vitamin de Gamborg (1 ml/l), Glucose (20/l), 2,4-D (2 mg/l), BAP (0.05 mg/l), Phytagel (2.2 g/l)	-	-	-
Expression (differentiation)	Expression of the new genetic program	Somatic embryo expression medium	DKW mineral complex Vitamin de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)

Source: Authors

(Table 2).

After preparation, all culture media were autoclaved at 121°C, under a pressure of 1 bar for 20 min (Sulzer autoclave).

Disinfection of flower buds

The immature flower buds harvested early in the morning (before 9 am) were marked according to genotype and transported in a cooler to the *in vitro* culture laboratory. These flower buds were then disinfected by immersion in a 1% (w/v) calcium hypochlorite solution, to which a few drops of tween-20 were added. After a contact time of 40 min with the 1% (w/v) calcium hypochlorite solution, the flower buds were rinsed three consecutive times with sterile distilled water at 2 min intervals.

Seeding and subculturing

Under the laminar flow hood, the disinfected immature flower buds were dissected with forceps and a scalpel blade in a Petri dish. Following this dissection, the petals explants and staminodes explants were isolated and used as explants.

Petals and staminodes explants of the five genotypes were grown simultaneously and separately in the four induction media at a rate of 25 staminodes explants and 25 petals explants per Petri dish. Each Petri dish was considered an experimental unit. However, for each genotype depending on the type of explant (staminode or petal), 20 experimental units were performed. The experiment was organized with a completely randomized design with two factors (genotype and explant type). Thus, a total number of 1000 explants (500 petals explants and 500 staminodes explants) per genotype and induction medium were used as explants for primary somatic embryogenesis. After seeding, the cultures were incubated in the dark (at 26 ± 1°C) for 14 days. At the end of the 14 days of incubation: (a) The cultures were transferred to a secondary callus induction medium and incubated for 14

days under the same conditions as before. After 14 days in the secondary callus induction medium, the explants were transferred to the embryo development medium (EDM) and the cultures were incubated for 28 days. At the end of the 28 days, five additional subcultures (at 28-day intervals) were then grown on EDM and incubated under the same experimental condition Protocol I).
(b) The cultures were transferred to the EDM and incubated for 28 days in the dark. Three successive transplants at 28-day intervals were then made on the same medium and cultures were incubated under the same conditions (protocols II, III, and IV). The test was replicated three consecutive times under the same experimental conditions.

Evaluation of responses to somatic embryogenesis

For each genotype, the percentage of callogenic explants and embryogenic callus is evaluated by counting the number of explants producing callus and the number of callus-bearing embryos. The parameters assessed are expressed by the formulae:

(1) Percentage of callogenic explants (PCE) (Garcia et al., 2016):

$$PEC=NEC/NTE\times100 \quad (1)$$

where NEC= number of explants yielding a callus and NTE= total number of explants grown.

(2) Percentage of embryogenic callus (PEC) (Garcia et al., 2016):

$$PCE=NCE/NEC\times100 \quad (2)$$

where NCE= total number of embryogenic callus and NEC= number of explants yielding callus.

(3) Number of embryos: The total number of embryos produced was counted every 4 weeks until 24 weeks on EDM medium. Also, the number of embryos produced is recorded per subculture time. The average number of embryos produced per genotype was calculated at each subculture.

(4) Embryogenesis efficiency (EE) (Garcia et al., 2016): EE values are a measure of the net efficiency of a given genotype at the various stages and conditions of culture. EE is calculated according to the formula:

$$EE=NPSE/NTE \quad (3)$$

where NPSE= number of primary somatic embryos produced and NTE= total number of explants grown.

Statistical analysis

Statistical analyses were performed using R software (Version 4.2.0). The experimental values obtained were all subjected to an analysis of variance (ANOVA). When the ANOVA indicated that at least one mean was different from the others, a post hoc Tuckey HSD or Kruskal-Wallis test at the 5% threshold was adopted to separate the means.

RESULTS

Morphologies of primary somatic embryos

Staminodes and petals explants of the five cocoa genotypes (C1, C8, C9, C14, and C16) produced calli in culture. Irrespective of genotype, calluses were observed between days 10 and 14 of culture in the callus induction medium in both experiments. In the embryo expression medium, callogenesis was followed by the differentiation of somatic embryos at different developmental stages (globular, heart, torpedo, and cotyledonary). However, some embryos showed malformations and others were translucent (Figure 1).

Effect of subculture time on response to somatic embryogenesis

Percentage of callus induced

The experimental results are presented in Figure 2. Analysis of this figure shows that all genotype explants (C1, C8, C9, C14, and C16) formed callus from the primary callus induction medium (CIM) and the callus development medium (CDM). The highest percentages, 100%, were obtained with petals explants of the C8 genotype. The lowest rate was observed in petals explants of the C1 genotype (87.13 ± 13.67%). On the other hand, significant differences in percentages were not observed between petals explants of C9 (99 ± 0.72%), C14 (99 ± 0.71%), C16 (96.5 ± 3.96%), C8 (100%), and staminodes explants of C1 (97 ± 0.82%), as well as C14 (98 ± 0.87%). Also, the percentages of staminodes explants producing calli from C8 (93 ± 2.5%) and C9 (93 ± 1.97%) were not significantly different from petals explants producing calli of C16 (88.57 ± 6.51%). Moreover, the percentages of calli derived from petals explants of C1 (87.13 ± 13.67%) and calli from staminodes explants of C16 (88.57 ± 6.51%) were not significantly different.

Percentages of embryogenic callus

The results obtained from the percentages of embryogenic callus of explants of different genotypes are as shown in Figure 3. The analysis shows that of all the callogenic explants, only the petals explants of genotype C1 produced the highest rate of embryogenic callus, that is, 25 ± 2.34%. The lowest rate was 0%, which was observed in C8, C14, and C16 staminodes explants. However, the embryogenic callus of C1 petals explants is significantly different from C8 (5 ± 3.13%), C9 (8 ± 1.76%), C14 (11 ± 3.09%), C16 (7 ± 2.56%) and C1 (7 ± 1.81%), C9 (5 ± 3.13%) staminodes explants.

Embryos produced

Figure 4 shows the average number of embryos produced per explant. The highest average number of embryos per explant was observed in petal explants of

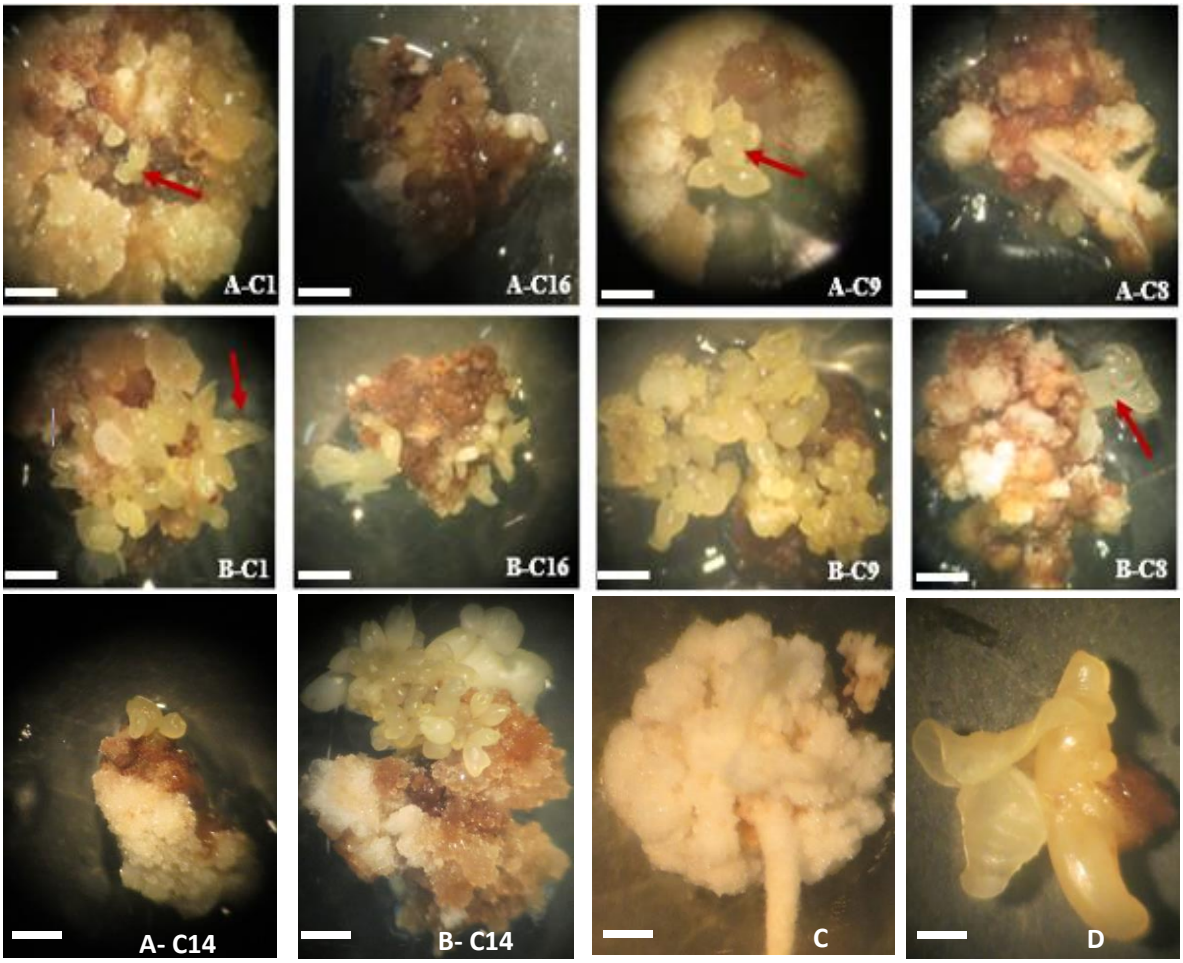

Figure 1. Primary somatic embryogenesis of *T. cacao* (beginning of somatic embryo productions of genotypes: C1 (A-C1), C16 (A-C16), C9 (A-C9), C8 (A-C8) C14 (A-C14). Maximum observed somatic embryo production of the genotypes: C1 (B-C1), C16 (B-C16), C9 (B-C9), C8 (B-C8) C14(B-C14). The red arrows show the different stages: globular (A-C1), heart (A-C9), torpedo (B-C1), and cotyledonary (B-C8). Non-embryogenic callus (C), translucent malformed embryo (D).
Source: Authors

the C1 genotype with a value of 28 ± 3.4. However, staminode explants of the C8, C14, and C16 genotypes did not produce any embryos. Thus, they constitute the lowest number of embryos. On the other hand, the petals explants of the C1 genotype are significantly different from the petal explants of C8 (4 ± 1.3), C9 (15 ± 3.3), C14 (17 ± 2.9), C16 (10 ± 2.1), and the staminodes explants of C9 (15 ± 3.3), C1 (4 ± 1.5).

Evolution of embryo production by subculture time

For this study, the evolution of somatic embryo production was assessed through the estimation of the number of embryos produced by subculture time.

The results obtained were presented in Figure 5. The analysis of the figure indicates that the petals explants of genotypes producing the most embryos from the beginning (2nd and 4th weeks) are C1 with an average of 2. While the genotypes of C9, C14, and C16 produce on average 1 embryo between the second and fourth weeks of subculture in the embryonic development medium (EDM). Embryo production was therefore rapid or early for these four genotypes. Furthermore, their number of embryos increased with subculture time (4 weeks interval) and reached a maximum between the 8 and 12th weeks of subculture with an average of 19 for C1, 7 for C9, 11 for C14, and 8 for C16. Thereafter, production decreased and stopped between the 16 and 20th weeks of subculture with an average of 3 embryos for C1, 2 for C9, 2 for C14, and 1 for C16. However, the first embryos of the C8 genotype appeared between the 12 and 16th weeks with an average of 2 embryos. The appearance of the first C8 embryos is therefore late compared to the

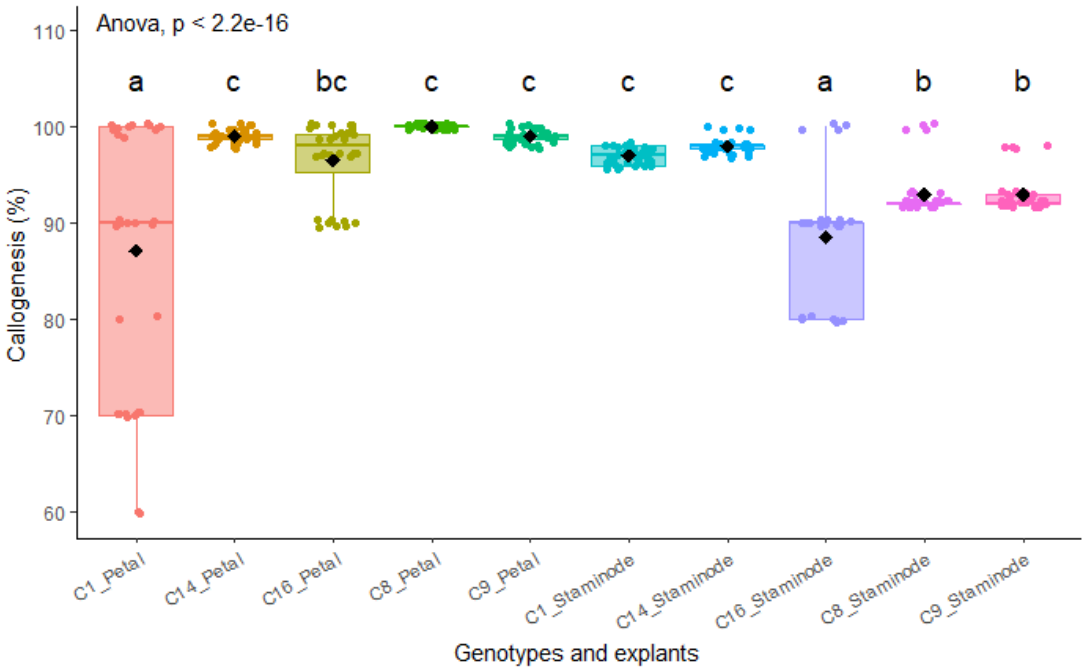

Figure 2. Percentage of callus induction in staminodes and petals of elite genotypes of *T. cacao*.
Source: Authors

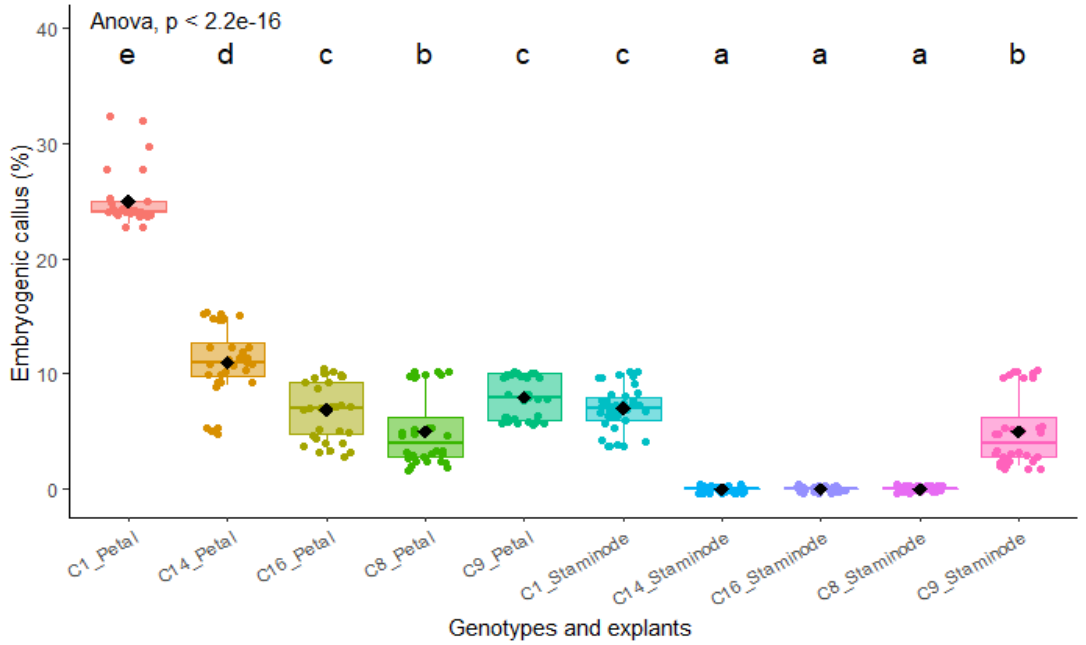

Figure 3. Percentages of embryogenic callus of elite genotypes of *T. cacao*.
Source: Authors

other genotypes. Nevertheless, the number of C8 embryos varied very little before reaching a maximum in the 16 and 20th weeks of subculture, with an average of 3 embryos. This production decreased thereafter and stopped between the 20 and 24th weeks of subculture.

The evolution of embryo production from staminode

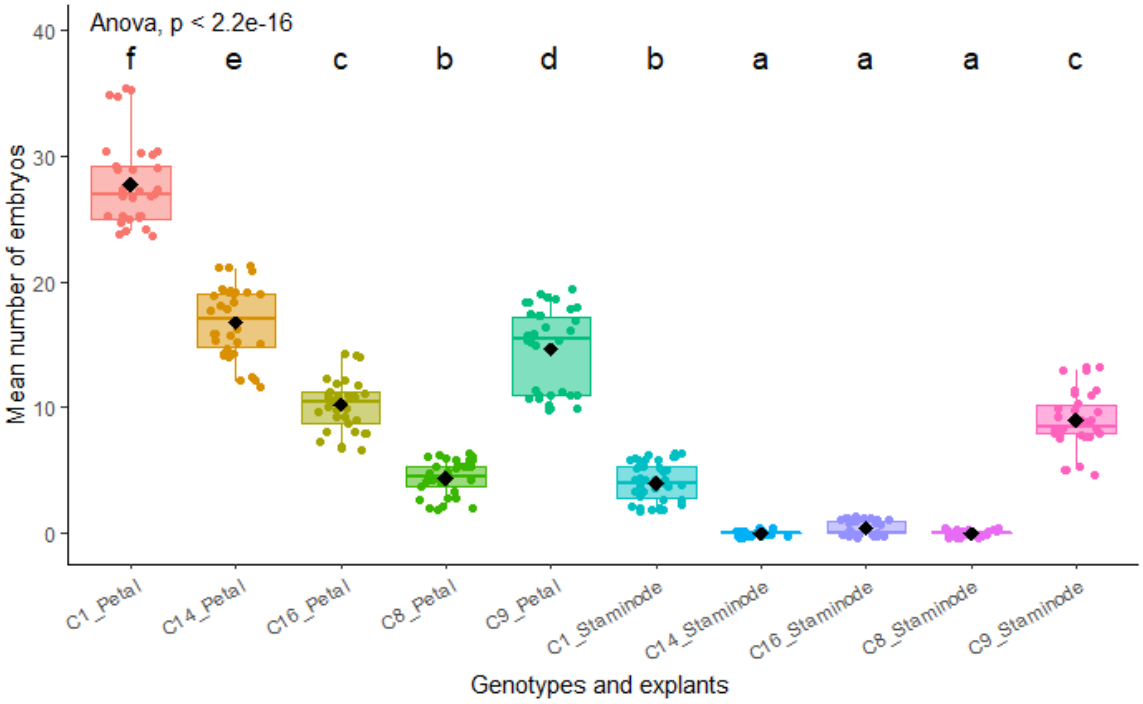

Figure 4. Number of embryos produced per explant according to elite genotypes of *T. cacao*.
Source: Authors

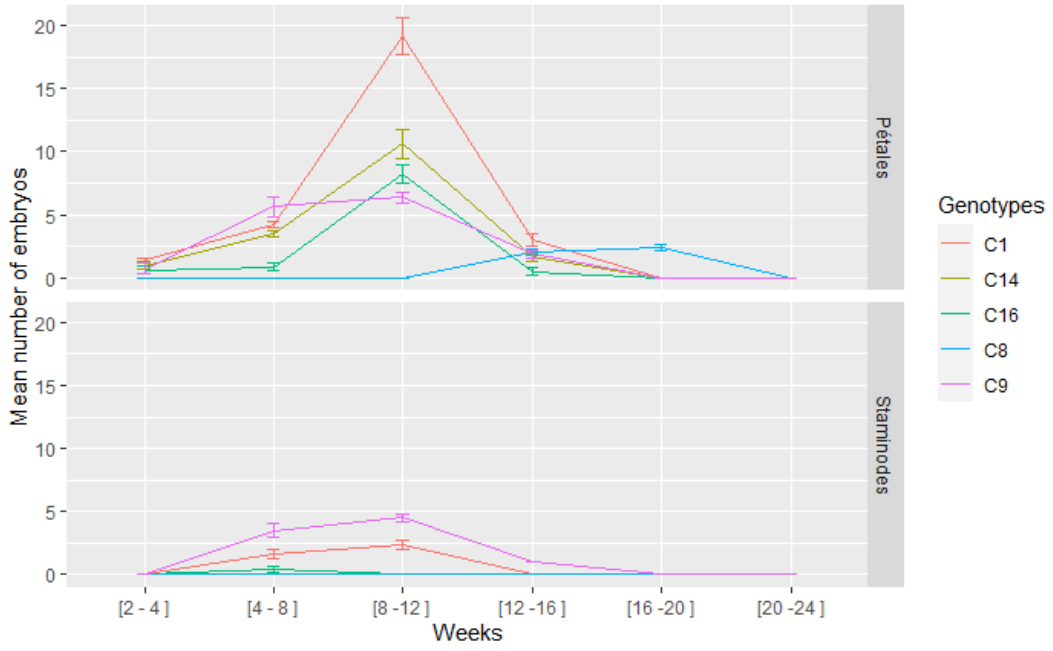

Figure 5. Evolution of embryo production according to subculture time of elite genotypes of *T. cacao*.
Source: Authors

explants was observed between the fourth and eighth week of subculture. The genotype that produced the most embryos was C9 with 4 embryos. During the same period, the C1 and C16 genotypes produced 3 embryos,

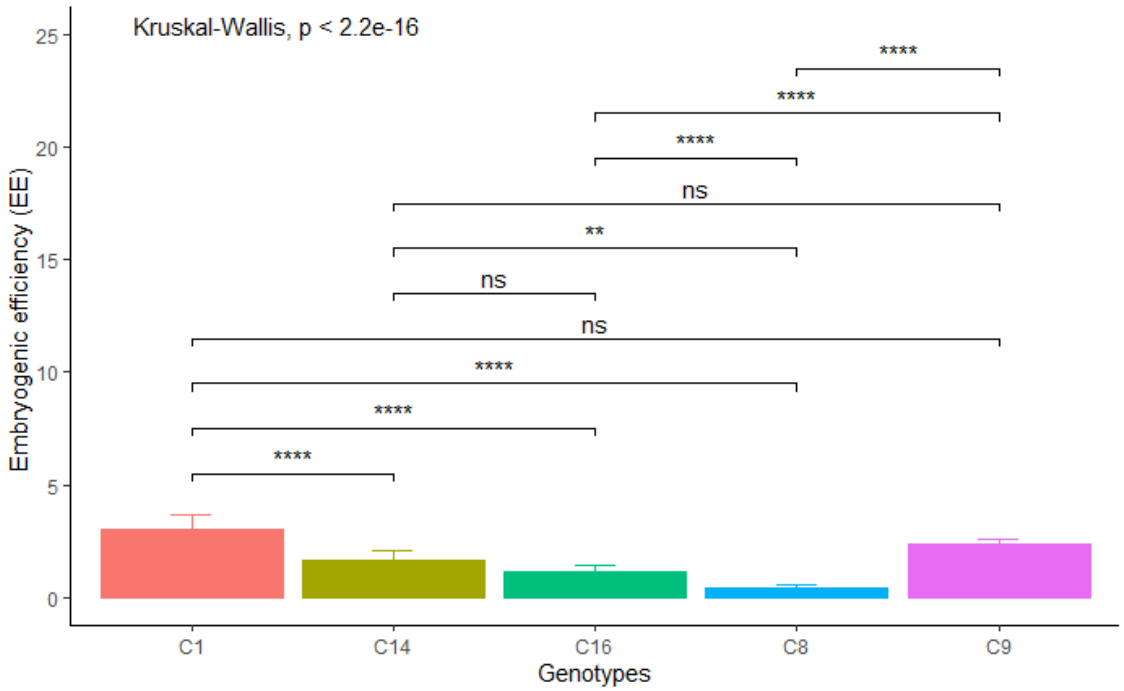

Figure 6. Somatic embryogenesis efficiency according to *T. cacao* elite genotype.
Source: Authors

respectively. Subsequently, embryo production increased for these genotypes and reached a maximum between the 8 and 12th weeks of subculture with average embryos of 3 for C1, 6 for C9, and 1 for C16. These embryo production values then decreased to zero between the 16 and 20th weeks of subculture.

The efficiency of somatic embryogenesis of elite genotypes

About the efficiency of somatic embryogenesis of the genotypes, the results were presented in Figure 6. The analysis shows that the most responsive genotype to embryogenesis is C1 with a somatic embryogenesis efficiency (EE) value of 3.1. In contrast, the least responsive genotype is C8 with an EE of 0.4. However, the EE value of C1 is significantly different from that of the C14 genotype with an EE of 1.7, and the C16 genotype with an EE of 1.1, except that of C9 with an EE of 2.4.

Effect of explant preferential response on somatic embryogenesis

Percentage of callus induced

Petals and staminodes explants were grown on DKW basal medium containing varying combinations of 2,4-D and kinetin. After 28 days of cultivation on the callogenesis induction and expression media, the results obtained were recorded in Table 3. The observed callus induction rate varied between 86 ± 18.6 and 100%. The most callogenic explants (100%) were observed with petals explants of C16, C8 and C9 genotypes for the initial culture media of protocols II, III, and IV. Similarly, staminodes explants of C16 genotypes for the medium of protocols II, C1, C14, C8, and C9 genotypes for culture media of protocols II, III, and IV were the most callogenic (100%). In contrast, the least callogenic explant was with C1 genotype petals explants (86 ± 18.6%). However, there were significant differences between the callogenesis of staminodes and petals explants of C1, C14, C16, C8, and C9 genotypes for the culture medium of protocols I, II, II, I, and IV.

Percentage of embryogenic callus

After 28 days of culture on callogenesis induction and expression medium, explants subcultured at 28-day intervals for 16 weeks on somatic embryo expression media recorded results shown in Table 4. All initial culture media (protocols I, II, III, and IV) did not allow all types of genotype explants to form embryogenic callus. Thus, the most embryogenic explant callus (54 ± 26.9%) was obtained with staminodes explants of genotype C9 for the culture medium of protocol III. As for the medium of protocol II, staminodes explants of C1, C14, C1, 6, and

Table 3. Percentages of callus formed from culture media of protocols I, II, III, and IV.

Genotype	Explants	Percentages of embryogenic callus			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	87 ± 13.5^{a}	86 ± 18.6^{a}	99 ± 1.7^{cf}	99 ± 1.7^{cf}
	Staminodes	97 ± 1^{b}	100^{f}	100^{f}	100^{f}
C14	Petals	99 ± 0.7^{c}	90 ± 22^{d}	96 ± 7.6^{d}	96 ± 7.6^{d}
	Staminodes	98 ± 0.9^{cb}	100^{f}	100^{f}	100^{f}
C16	Petals	97 ± 3.9^{b}	100^{f}	100^{f}	100^{f}
	Staminodes	89 ± 6.4^{d}	100^{f}	97 ± 6.5^{b}	97 ± 6.5^{b}
C8	Petals	100^{f}	100^{f}	100^{f}	100^{f}
	Staminodes	93 ± 2.5^{e}	100^{f}	100^{f}	100^{f}
C9	Petals	99 ± 0.7^{cf}	100^{f}	100^{f}	100^{f}
	Staminodes	93 ± 1.7^{e}	100^{f}	100^{f}	100^{f}

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

Table 4. Percentages of embryogenic callus formed from culture media of protocols I, II, III, and IV.

Genotype	Explants	Percentages of embryogenic callus			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	25 ± 2.3^{a}	21 ± 10.5^{a}	10 ± 5.1^{b}	7 ± 8.9^{b}
	Staminodes	7 ± 1.8^{b}	0	10 ± 2.8^{b}	21 ± 10.33^{a}
C14	Petals	11 ± 3.1^{c}	10 ± 6.1^{b}	0	0
	Staminodes	0	0	11 ± 4.2^{c}	14 ± 5.4^{d}
C16	Petals	7 ± 2.5^{b}	10 ± 5.1^{b}	6 ± 3.4^{b}	0
	Staminodes	0	0	6 ± 5.2^{b}	12 ± 7.7^{c}
C8	Petals	5 ± 3.1^{d}	0	0	0
	Staminodes	0	0	0	0
C9	Petals	8 ± 1.7^{e}	7 ± 5.3^{c}	6 ± 2.1^{b}	7 ± 5.3^{b}
	Staminodes	5 ± 3.1^{f}	0	54 ± 26.9^{d}	6 ±2.8^{e}

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

C8 genotypes did not form embryogenic callus. Also, the petal explant callus of the C8 genotype was not embryogenic for the culture media of protocols III and IV.

Embryos produced

The results of the embryo production are shown in Table 5. Analysis of this table shows that all genotypes produced embryos from the culture media (protocols I, II, III, and IV) tested. However, significant differences were observed depending on the genotype and the type of explant used. Thus, the culture medium according to genotype and explant that produced the most embryos (88 ± 56) was the culture medium of protocol III, C9 genotype and staminodes explants. On the other hand, for the culture medium of protocol II, the petals explants of C1, C14, and C16 genotypes produced more embryos

Table 5. Number of embryos produced per explant from culture media of protocols I, II, III, and IV.

Genotypes	Explants	Number of embryos per explant			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	19 ± 4^{a}	24 ± 23^{a}	5 ± 3^{b}	0
	Staminodes	2 ± 1^{c}	0	5 ± 1^{b}	13 ± 3^{d}
C14	Petals	11 ± 3^{e}	17 ± 9^{b}	5 ± 2^{b}	0
	Staminodes	0	0	4 ± 3^{b}	9 ± 0.0^{g}
C16	Petals	8 ± 2^{f}	18 ± 8^{b}	3 ± 2^{c}	0
	Staminodes	0	0	2 ± 1^{c}	6 ± 4^{h}
C8	Petals	2 ± 1^{c}	0	0	0
	Staminodes	0	0	0	0
C9	Petals	6 ± 1^{b}	4 ± 2^{c}	3 ± 1^{c}	0
	Staminodes	5 ± 1^{b}	0	88 ± 56^{i}	4 ± 1^{b}

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

than the other media (protocols I, III, and IV). Similarly, for the protocol IV culture medium, staminodes explants of C1, C14, and C16 genotypes produced more embryos than the protocols I, II, and III culture media. However, the C8 and C9 genotypes produced more embryos with petals explants from the protocol I medium.

DISCUSSION

Explant responses to somatic embryogenesis in cocoa are genotype-dependent. To select a pool of genotypes associated with somatic embryogenesis performance, five genotypes (C1, C16, C9, C14, and C8) were tested on different culture media. The results in the study of the effect of subculture time on somatic embryogenesis response showed that petals and staminodes explants of C1, C8, C9, C14, and C16 genotypes induced callus at rates ranging from 87.13 to 100%. Similar observations were made by Kahia et al. (2017) and Osorio Montoya et al. (2022). According to their reports, *T. cacao* flower bud explants yield high percentages of callus during cocoa somatic embryogenesis. Our results show a success that could also be explained by the nutrient composition of the culture medium and the effect of the hormonal balance used (Daouda et al., 2019; Osorio Montoya et al., 2022). The hormonal balance (2,4-D / TDZ) used in the culture medium is the best condition to obtain callus (Boutchouang et al., 2016). Indeed, auxin (2,4-D) and cytokinin (TDZ) facilitate cell division and protein synthesis that affects callus and somatic embryo formation (Garcia et al., 2016). In contrast, the significant differences between the callus percentages of the different genotypes would be due to a genotype effect. This can be explained either by the concentration of endogenous phytohormone of the explant type of genotypes such as indole-3-acetic acid or by the sugar concentration of the explant which influences the callus formation process (Grzyb et al., 2017).

Furthermore, calli from petals explants of the C1 genotype were found to be more embryogenic than calli from petals explants and staminodes explants of other genotypes. Similar results were reported in the somatic embryogenesis of *T. cacao* by Garate-Navarro and Arévalo-Gardini (2017), in which all explants gave callus. However, not all were able to produce embryogenic calli. These observed differences in response could be explained either by the physiological state of the explant donor tree or by the accumulation of ethylene and carbon dioxide produced in the dark by the plant tissues in the Petri dishes during *in vitro* cultivation, which would hinder cell growth and regeneration (Boutchouang et al., 2016). Likewise, excessive hydrogen peroxide (H_2O_2) accumulation in calli may lead to the loss of embryogenic potential of calli (Peng et al., 2020). This variability in the response to somatic embryogenesis would therefore also be attributed to the effect of genotypes. However, the influence of genotype in the response to somatic embryogenesis in cocoa has also been reported in several previous works (Kouassi et al., 2017; Dangou et al., 2002).

The study carried out here on the evolution of embryo production according to the time of subculture of the genotypes has made it possible to highlight the effect of subculture time on the response to somatic embryogenesis of *T. cacao*. Indeed, there is an early

production of embryos by the C1, C9, C14, and C16 genotypes between the 2nd and 4th weeks in the EDM medium, and a late production of embryos by the C8 genotype between the 12 and 16th weeks in the EDM medium. These results may be linked to the plant gene, which has an immediate impact on the recalcitrance of certain tissues and genotypes concerning their totipotency (Daouda et al., 2019). Prolonged subculture on the EDM medium of *T. cacao* is, therefore, necessary to overcome recalcitrance or improve the response of certain genotypes. Indeed, according to Ren et al. (2022) during *in vitro* culture, the long-term subculture of some plant genotypes, the high endogenous content of cytokinin like indol-3- acetic acid in tissues cellular is beneficial to the vigorous growth of embryogenic calli and the high potential for somatic embryogenesis. In addition, the efficiency of somatic embryogenesis showed that the C1 genotype had a better production of embryos than the C14, C16, C8, and C9 genotypes. These results would also reflect the strong dependence on the genotypic effect. The genotypic differences observed in the somatic embryogenesis response probably reflect either endogenous soluble sugar provides indispensable energy as a carbon source, either genetic variation in the concentration of endogenous phytohormones, or the type of endogenously produced compounds such as polyamines, ethylene, phenolic compounds, auxin, or the synergy of endogenous phytohormones with exogenous phytohormones supplied in a medium (Ren et al., 2022; Peng et al., 2020).

Furthermore, the study carried out on the preferential effect of explants on somatic embryogenesis response identified media suitable for somatic embryogenesis responses of elite genotypes. Indeed, the production of embryos from C1 (petals explants), C14 (petals explants), and C16 (staminodes explants) was most effective in protocols II (C1 and C14) and IV (C16). Additionally, protocol I (with staminodes explants) and protocol III (with petal explant) were found to be suitable to obtain embryos from C9. Concerning the C8 genotype, the production of embryos is better only with petals explants in the culture medium of protocol I. The variation in response observed between genotypes would confirm on the one hand the differences in the requirements of endogenous and exogenous phytohormones of the explants of genotypes, and on the other hand, the endogenous storage substances (protein, sugar, and starch) in the explants. In fact, the molecular components (phytohormones, sugar, protein, lipids) in plant organs are influenced by their genetic origin, physiological status, and environmental factors. Environmental changes affect various biochemical reactions, often disrupting the balanced distribution of metabolites in cells (Shah et al., 2019). Moreover, The results of the present study are in line with those of Garcia et al. (2016) who showed that any variation in endogenous auxin levels would likely impact embryogenic capacity and other underlying response variations from one genotype to another. Also, Peng et al. (2020) showed that endogenous components like soluble protein, starch, soluble sugar, and superoxide dismutase were involved in the development of embryogenic calli in the pin. Their results indicate that somatic embryogenesis involves energy storage, and antioxidant enzymes cooperate to regulate the occurrence and development of embryos. This discovery of protocols adapted to genotype pools *in vitro* culture of *T. cacao* could be used for large-scale production for commercial purposes.

Conclusion

The first step of this study was to assess the effect of subculture time on the response to somatic embryogenesis. It was found that among the five genotypes tested, the C1, C9, C14, and C16 genotypes produced embryos early while the C8 genotype produced embryos late. Also, the C1 genotype has a higher somatic embryogenesis efficiency than the C8, C9, C14, and C16 genotypes.

The second step was to test the preferential effect of explants on embryo production and showed variability of results concerning the tested genotypes. Indeed, staminodes explants of the C1, C14, and C16 genotypes preferred the culture medium of protocol IV, while their petals explants preferred the culture medium of protocol II. Whereas, staminodes explants of the C9 genotype prefer the culture medium of protocol III and its petals explants prefer the culture medium of protocol I. As for the C8 genotype, its petals explants prefer the culture medium of protocol I. However, its staminodes explants did not produce embryos. This study, therefore, highlighted the somatic embryogenesis response pool of the five elite genotypes of *T. cacao*.

ABBREVIATIONS

2,4-D, 2,4-Dichlorophenoxyacetic acid; **TDZ,** thidiazuron; **EE,** efficiency of embryogenesis; **PEC,** Percentage of embryogenic callus; **PCE,** Percentage of callogenic explants; **KIN,** kinetin; **DKW,** Driver and Kuniyaki; **CIM,** primary callus induction medium; **CDM,** secondary callus development medium; **EDM,** somatic embryo development medium.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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