Encapsulation of Brunfelsia pauciflora microprogated shoots as a suitable conservation protocol

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Abstract

The current study aimed to establish an efficient *in vitro* propagation and conservation protocol for *Brunfelsia pauciflora* plant. The effect of plant growth regulators types (BAP, Kin, 2ip and NAA) and concentrations at 0.0, 1.0, and 2.0 mg/l were studied during establishment phase in comparing with hormonal-free medium. *In vitro* proliferated shootlets were encapsulated using various levels of sodium alginate at 1, 2 and 3% (W/V) and calcium chloride at 2.5,5 and 10 %. Regrowth ability of encapsulated explants was evaluated after conservation for 3, 5, and 7 weeks and compared with non-encapsulated buds. The findings of the current study proved that during establishment phase, applying BAP at 2.0 mg/l gave the best results during the three successive subcultures. The best treatment 2ip (2.0mg/l) + BAP (2.0mg/l) + NAA (2.0mg/l) that was considered ideal propagation protocol for shooting and rooting of *Brunfelsia*. Successful plant recovery from encapsulated explants using low concentrations of sodium alginate (1%) and calcium chloride at 2.5%, proved that the explants could to be survive at highest percent (100%) during conservation periods 3, 5 and 7weeks. Increasing calcium chloride to 5% with the same concentration of sodium alginate (1%) caused gradually reduced percent of survival to 53.33% with increasing conservations period (from 3 to 7weeks). It is the first paper of the application of syndetic seed technique on *Brunfelsia* for the shortest period conservation and successive renovation of shootlets whose genetic stabilization was evaluated by analysis of RAPD that proved that the genetic similarity ranged from 56.1 to 80%. These results were confirmed by anatomical sections made in *Brunfelsia* leaf blade.

Introduction

Brunfelsia pauciflora is a flowering species belongs to *Solanaceae* family. Itis an evergreen shrub native to Brazil. It's known as many names as Brazil rain tree, morning-noon and night and yesterday-today-and-tomorrow. It is blooming from spring and fall but it may repeat bloom anytime during the year under adequate conditions. The high ornamental value of this plant is due to the obvious color change of the flower (Vaknin *et al*. 2005). After the flower opening at 2 to 3 days the petal colour begins to convert gradually from deep purple to white color.These are changes related to the types of pigments especially reduced anthocyanin content (Michal, 2009). Broadly, the pigment of petals is spread in the differint tissues as the vacuoles of epidermal cells and cell walls (Markham *et al*. 2000), palisade, and chromoplasts (Kay *et al*. 2008). Like the *Solanaceae* members. Brunfelsia tree was drought tolerant and attractive as a dwarf potted plant so it is the most distributed in the warmer sites.

Brunfelsia pauciflora is the only one species from *Brunfelsia* genus grown indoors, propagates in spring when tip cuttings of new growth are available. New growth is produced in four to six weeks. There are some problems when planted *Brunfelsia* in field such as Pale or yellow leaves result from potting mixture that is not acid enough. Also, weak growth is a sign of aphids, which suck the sap of the plant. Some authors reported that tissue culture is considered an alternative to traditional methods for propagation of several species using various tissues (Wang *et al*. 2008; Sakr *et al.* 2017).

Encapsulation has been used as biotechnology for many fields as horticultural, aromatic and medicinal plants (Murch *et al*. 2004; Rai *et al*. 2009). Furthermore, this technology is too hopeful with respect to the progression of the micropropagation and in vitro preservation for many ornamental species plants (West *et al*. 2006; Ozden-Tokatli *et al*. 2008), particularly when used commercially on a large scale. It is playing an affective role in mass propagation for species of varieties distinguished by depressed reproduction rates and decline rooting. It can be applied to produced disease free clones and conserve gene pools.

Synthetic seeds can be formed from the different tissues that non-embryogenic as shoot tips, nodal segments, protocorm and portion of callus to develop into whole plantlets (Standardi and Micheli, 2013). In addition, encapsulation, non-embryogenic micropropagules are comparatively cheap to reproducetion and easy to handle, transport and plant (Rao *et al*. 1998). Synthetic seeds have been studied in numerous plants species with successful (Ray and Bhattacharyaa 2008; Singh *et al*. 2009; Saha *et al*. 2014). The synthetic seed- derived plantlets are still unbeknown the gentetic stability except recent report (Gangopadhyay *et al*. 2005). Also, the anatomical changes in the plants after recovery are few in the literature.

So, the aim of this study was to test different sodium alginate and calsium chloride concentrations on conserved nodel segments of *Brunfelsia pauciflora* for various storage periods to develop a suitable conservation protocol by encapsulation

regenerated shoot buds, checking their genetic stability using PCR analysis and studing anatomical changes after recovery.

Materials And Methods

Plant material and culture condition: The Brunfelsia shurb planted in green house of National Research Centre was source for explants that are used in this experiment. Shoot explants were prepared by rinsing directly below running tap water and little drops of liquid soap for 1hour. Explants rinses for 4 times with distilled and sterilized water, then explants were surface sterilized in 70%(v/v) ethanol for3 minute under an aseptic condition in laminar air-flow cabinet, fallowing by 15% commercial sodium hypochlorite solution and one drop of commercial sodium and one polyoxy ethylene sorbitonmonol aurate (tween 20) for 5 to 6 min. Finally, they were flooded in 0.1% mercuric chloride with a few drops of tween -20 for 2 to3 min. After that, they were immersed in 0.1% mercuric chloride (MC) with a few drops of tween-20 for 2-3 min. After that, they were rinsed three times with sterile distilled water. Explants were then grown on growth regulator free MS-medium (Murashige and Skoog, 1962), 25g/l sucrose and 8.0 g/l agar. The medium was adjusted to pH 5.7 ± 0.1; with 25 ml in 200 ml capacity glass jars before autoclaving at 121C° and 1.2 kg/cm^2 for 15 min. All cultures were incubated at 23c°±2c° under 16-h photoperiod provided by fluorescent white light 30µmol m^{-2}s^{-1}.

***In vitro* culture establishment:**

Shootlets proliferation: After two months from starting stage, free contamination shoots were regenerated on MS-culture medium containing various cytokines [6-benzyl amino purine (BAP), and Kinetin (N-6-fouryl adenine) (Kin)] at different concentrations (0.0, 1 and 2mg/l). All concentrations were replicated three times with three explants per replicate. The obtained shoots were repeat subcultured three times each 45 days. Data contain shoot number; shoot length and number leaves per shootlet were recorded for each subculture.

Optimization of micropropagation culture medium: To optimize the suitable micropropagation medium, shootlet were cultured on MS medium supplemented with 2g/l active charcoal and different growth regulators concentrations [6-benzyl amino purine (BAP) at 2mg/l, 6-γ, γ-dimethl ally amino purine riboside (2ip) at (1 and 2mg/l) Indole-3- butyric acid (IBA) at (0.4 and 0.8mg/l) and Naphthalene acetic acid (NAA) at (2 and 4mg/l). Each treatment was replicated three times with three explants per replicate. The obtained shootlets of established cultures on optimal MS medium that containing 2 mg/l 2ip+ 2mg/l BAP+ 2mg/l NAA and 2g/l of active charcoal were used as explants for conservation using encapsulation in this study. Nodal segments, approximately 1 to 1.5 cm long taken from in vitro proliferation shootlet cultures were encapsulated aseptically.

***In vitro* conservation using encapsulation:**

Preparation of sodium alginate and calcium chloride: It was added in three concentrations of alginate (1, 2, and 3% W/V) to1/2 MS liquid medium with 25 g/l sucrose and then autoclaved for 20min at 121/C° at 1kg/cm^2 and incubated for three days. Calcium chloride was prepared at three concentrations (2.5, 5, and 10 %) and was dissolved in 100ml sterilized distilled water and then sterilized into autoclave. Treatments described as Control shoot explants that non-capsulated (Con.); 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water(A1); 1% Sodium alginate + 5g Calcium chloride/100ml distilled water (A2);1% Sodium alginate +10g Calcium chloride/100ml distilled water) (A3); 2% sodium alginate+ 2.5calcium chloride (b1); 2% sodium alginate + 5g Calcium chloride/100ml distilled water(b2); 2% sodium alginate+ 10g/ Calcium chloride/100ml distilled (b3); 3%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water(C1); (3% Sodium alginate + 5g Calcium chloride/100ml distilled water (C2) and 3% Sodium alginate + 10g Calcium chloride/100ml distilled (C3) during different conservation periods for 3, 5, and7 weeks.

Encapsulation of shoot explants: Nodal segments (1 to 1.5 cm) were separated from *in vitro* stock cultures of *Brunfelsia pauciflora* for encapsulation, the explants were plunged into various concentrations of sodium alginate solution supplemented with MS medium. Drops of alginate solution with shoot nodal segment were dropped into sterile plastic plate wells that were immersed in 100 mM calcium chloride (CaCl2·2H2O) for complexion for 1 to 2 min. All operations were performed under sterile conditions, and then placed in sterilized glass jars each jar has 3 capsulations with three replications. All encapsulated cultures

preserved at 24c° under 16 hours photoperiod provided by fluorescent white light 30µmol $m^{-2}s^{-1}$ in light for different three times (3, 5, and 7weeks).

Explants recovery: At the end of each conservation period i.e., 3, 5, and 7 weeks of encapsulated explants, cultures were transplanted onto optimization MS culture medium with sucrose (25 g/l), 2g/l of active charcoal, 2mg/l of 2ip, 2mg/l BAP and 2mg/l of NAA for two months. Each jar has three encapsulated explants. After two months from culture, the *in vitro* morphological parameters were recorded as: survival %, shootlets number, shootlets length (cm) and leaves number per explants.

Genetic Stability Assessment Using RAPD analysis:

DNA extraction and isolation from leaves: Genomic DNA was extracted from leaves *in vitro* survival plantlets which were encapsulated for 7weeks and DNA was isolated according to Dellaporta *et al*. (1983).

Randomly amplified polymorphic DNA-RAPD: It was carried out according to the procedure described by Hussein *et al*. (2006).A set of six random 10-mer primers were selected (Table1). These primers were synthesized at Metabion interational AG, Germany. Only six RAPD primers (OP-A 10- OP-A 13, OP-C 12, OP-D 07, OP-M 09, OP-Y-09) revealed discernible polymorphism among the control treatment. The amplification reaction was executed in 25µl total volum including 1xPCR buffer,1.5mM MgCl2, 2mM dNTPs,2.5U Taq DNA polymerase (all reagents from Promega Corp., USA),10mM primer and 25ng template DNA PCR amplification was done in a Biometra thermal cycler (Biomedizinische analytik gmbh).The amplification was programmed at 94Cº for 5 min as an initial denaturation cycler,followed by 35 cycles comprised of denaturation step at 94c°for 1min, an annealing step at 36c° for 1min, then an extension step at 72c° for 1min,the primer extension step was extended to 7min at 72c° in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5µg/ml)in 1x TBE buffer at 95 volts.A1kb DNA ladder (Fermentas GmbH,Germany) was used as molecular size atanderd.

Table 1 Sequence of the 18 decamer arbitrary primers assayed in RAPD-analysis to detect polymorphism among nine wheat parental genotypes

RAPD bands were recorded visually on the grounds of their presence (1) or absence (0), separately for each individual genotype against each primer. The scores obtained using all primers in the RAPD analysis were then collected for constructing a single data matrix. It was used to evaluated the genetic similarity and for constructing a UPGMA (un weighted pair group method of arithmetic means) dendrogram using computer program POPGENE (Version 1.31) (Yeh *et al*.,1999). The similarity index (SI) values between the RAPD profiles of any two individuals on the same gel were counted from RAPD marker according to the formula of Nei and Li (1979).**Similarity Index (SI)=** 2Nxy/(Nx + Ny).Where, N x y is the number of RAPD bands shared among the individuals x and y, Nx and Ny are the number of bands in individual x and y, respectively.

Anatomical studies: Leaves of conserved *Brunfelsia pauciflora* plantletsusing encapsulation for seven weekswere used aftertwo monthsof recovery and fixed in FAA (Formalin (10ml), ethyl alcohol70% (85ml) and glacial acetic acid (5ml). The fixed materials were then dehydrated and staining according to (Nassar and ElSahar, 1998).

Statistical analysis : The data were analyed using analysis of variance ANOVA and treatments' means were compared for significance by Duncan's New Multiple Range test at 0.05% level of probability (Duncan,1955) using COSTATV-63.

Results And Discussion

Shootlets proliferation

Microshoots (1.5 to 2cm in length) were *in vitro* repeat cultured three times on MS medium cantaining three levels of BAP (0.0, 1.0, 2.0 mg/l) alone or in combination with kin at the same concentrations (Table 2 and fig.1). The best treatment for most recoreded characters was BAP at 2mg/l during all recorded subcultures such as the number of shootlets per explants (14.67, 16.00, and 11.67shootlet/explant) and number of leaves per shootlet (37.33, 42.33, and 39.00), respectively. BAP (2mg/l) + Kin

(1mg/l) also caused relatively proliferated shoots (14.00 and 10.33 shootlet/explant) during the second and thrid subculture and number of leaves per shootlet (26.33, 44.33, and 28.67 leaf/ shootlet), respectively. In respect of shootlets elongation, MS free media (control) recorded the longhest shootlet (2.16cm) during the first subculture, then the reduced elongation was noticed garudaly to 2.00 cm with BAP at 2mg/l+ 2mg/l of Kin during second subculture.While, 2mg/l of BAP alone recorded shortest shootlet (1.83cm) during thrid subculture.

These results were agreed with previous studies such as Liberman *et al*., (2010) who mentioned that BAP (4.44µM) was the best treatments for *Brunfelsia calycina* proliferation. Sakr *et al*.(2017) on *Brunfelsia pauciflora* found that MS medium added 1mg/l BA blus 2 mg/l kin improved multuplication of microshoot that were repeated three times. Ali (2017), indicated that the highest values of number of shootlets of *Eustoma grandiflorum* /explant and number of leaves/shootlet were resulted from BA at 0.4mg/l, and the same concentration (0.4mg/l) of Kin gave the longest shoots as compared to control. Zenna (2020) on *Prunus cerasifera* Ehrh noticed that applying BAP at 1.5 or 2.0 mg/l produced the highest significant shootlets multiplication rate which was reduced insignificantly from 1st to 2nd subcultures and significantly from 2nd to 3rd subcultures when BAP was supplied at 1.0 mg/l to the culture medium.

The present study indicates the stimulation effect of BAP as cytokinin on proliferated shoots to establish an *in vitro* culture of Brunfelsia as was conformied by previous studies (Lattoo *et al*. 2006) who observed the effectiveness of BAP on shoot multiplication which was remarkalable but, less effective on shoot elongation compared with Kin and 2ip that improved the *in vitro* shoot elongation. This may be explained that cytokinins have a significant function for stimulating cell division and cell prolongation, throughout activating RNA synthesis, tonic protein synthesis and enzyme action (Kulaeva, 1980).

Optimization of micropropagation culture medium

Data presented in Table 3 exposed that, the highest values of shootlets number/explant (6.33 to 6.67 shootlet /explant) were produced when 2ip (1mg/l) + BAP (2mg/l) added with IBA at 0.4 or 0.8mg/l were used in MS culture medium. While, the longest shootlet (9.00cm) were resulted in doubled concentration of 2ip (2mg/l) which also gave the highest number of leaves (30.33 leaf / shootlet) as compared to control (MS free of hormone). In respect to rooting ability, it could be noticed that when shootlets were treated with 2ip at 2mg/l+ BAP at 2mg/l +NAA at 2mg/l gave the maximum values for rooting percentage (100%), number of roots (2.67 root /shootlet) and root length (6.26cm) comparing to control and other treatments as shown in (Table 3 and Fig.2). Our results are in agreement with those obtained by Shadparvar (2012) on *Cymbidium orchid* var 'Red Tiffani' mentioned that, the maximum number of shoots was obtained from medium containing 2mg/l 2ip, but the lowest number was obtained from medium which containing 1mg/l 2ip. While the longhest shoot was obtained from MS medium containing 4mg/l 2ip. Zenna and Taha (2018) on *Antigonon leptopus* suggested that the maximum number of shoot per explant was recorded with BAP at 2.0mg/l or BAP at 0.5mg/l + IBA (2.0mg/l) while, the longest shootlets were obtainend from 2ip 0.5 or 1.0 mg/l each combined with 0.2 of IBA Sparzak *et al*. (2019) found that the highest number of shoot was resulted from MS medium containing IBA at 0.5mg/l plus 1.0mg/l of BAP. Zenna (2020) mentioned that applying 2ip at concentrations over 0.5 mg/l produced cell expanding and therefor shootlet elongation and induced leaves formation of *Prunus cerasifera* Ehrh to gave the highest significant values. The best rooting percent (100%) was formed when shootlet was treated with 0.4 mg/l of IBA. It may be concluded that cytokinins are varye in their uptake, realization and mode of action. Cytokinins could be classified into two groups: highly effective group (BA), being more active, i.e. more multiplication rate, and weakly effective group (2iP and Kin) exhibited weak effects on multiplication rate.

Concerning rooting behavior, Sakr *et al*.(2017) noticed the improved roots formation when transferred shoots of *Brunfelsia pauciflora* on culture medium supplying with 2.0mg/l IBA and 3.0g/l activeated charcoal. Kaviani et al. (2018) found that the maximum root initiation and development was formed from MS medium adding (3.00mg/l BA plus NAA at 0.20mg/l). The longest root was recorded with the combination of (3.50 mg/l BA + 0.20 mg/l NAA).

Encapsulation of shoot explants

Several concentrations of encapsulation agents as sodium alginate or calcium chloride were tested to determine the perfect concentrations and the best period conservations furthermore, to obtained the best size and form of capsule or beads of

Brunfelsia paucifolra.

Recovery of encapsulated shootlets:

The explants started to penetrate wall of capsulate (the early wall capsule break and sprout) after 3weeks of incubation with some treatments (fig.3, 4). When the lowest concentration of material encapsulation agent as sodium alginate at 1% and calcium chloride at 2.5% (A1) was used, the explants could be survived at highest percent (100%) during conservation for 3, 5 and 7weeks. Increasing calcium chloride to 5% with the same concentration of sodium alginate (1%) (A2) caused gradually reduced percent of survival to 53.33% with increasing conservations period (from 3 to 7weeks). On the other hand, Using high concentration of sodium alginate at 3% and low concentration of calcium chloride at 2.5% (C1) gave the highest survival rate (100%) for shortest period conservation (3weeks) while, extending the conservation period to 7weeks led to gradual decreasing of survival rate (from 100 to 22.11%) as compared to control treatment that recorded the highest one (100%) with all period preservation as shown in (fig.3). Finally, both high concentrations of alginate (2 and 3%), and calcium chloride at (10%) (B3 and C3) caused the death of whole explants. Similarly, Bekheet (2006) noted that using 3 % of sodium alginate recorded the highest percentages of survival and development of bulblets encapsulated to plantlets and with high concentration (4 %) of sodium alginate; a capsule was so hard and prevented bulblets proliferation. Sakr *et al*. (2018) mentioned that increasing calcium chlorid concentrations from 1.1 to 3.3g/100ml led to significantly increase the mortality percentage, also the percentages of inhibition and sprouting were decreased by increasing of capsulation agents and period conservation. The longest period (8weeks) gave the highest percentage of inhibited shoot tip and gave the lowest sprouting (%).

The synergistic effect of alginate and calcium treatments led to divergent responses. All encapsulation agents have an important role in complexion and capsule hardness (Redenbaugh *et al*. 1991). The capsule gel is considered the source of nutrients that could assist the survival or accelerate the growth of explants, while a high level of alginate may inhibit the emerging shoots. From this study based on regrowth percent of shoot explants, it can be conclude that optimal concentrations were 1 and 3% of sodium alginate and 2.5 and 5% of calcium chloride which caused the best regrowth ability of encapsulated shootlet (100%) and this proved to be the most effective concentrations for resulting reasonably the optimal size, form and quality of beads or capsule *Brunfelsia paucifolra*. The low concentration of alginate solutions and calcium chloride solutions formed capsule that was fragile and difficult to transferee by forceps, while the rounded beads or a pear shape were produced when capsulated explants at high concentrations of sodium alginate (3%v/w) and calcium chloride at 10 g/ 100 ml (C3) but, this treatment caused death of explants. Encapsulation of explants was effected by sodium alginate and calcium chloride levels and period preservation, which with each other can be formed capsule their ideal properties. Rai *et al*. (2009) mentioned that both capsulation agents considered best combinations for complexion; additionally however, they are inexpensive, non-damage and easy to use and produced rising propagules to explant conservation. Our results are in agreement with Faisal and Anis (2007) on *Tylophora indica* ; Alatar and Faisal (2012) on *Rauvolfia tetraphylla* they found that sodium alginate at 3% (w/v) with100 mM of chloride calcium formed firm and chloride calcium formed solid,pure and regular beads during 30 minates an ion exchange. High levels of sodium alginate 4% or 5% were resulted Too hard beads and caused a delay in emerging of shoot, while capsule formed using low concentration of alginate (1, 2 or 3%) with calcium chloride at (25, 50mM) were too fragile to handle. Attia *et al*. (2018) prepared the Sodium alginate at 3, 4, and 5% (w/v) and treated the axillary buds which taken from *Rosa damascena trigintipetala* Dieck. They noticed that the fast wall break of the capsule during 5 days and all capsules sprouted 100% after 10 days from control treatments and sodium alginate at 3% plus to 75 CaCl2. Increasing CaCl2 to 100mM with 3% of SA led to delay wall capsule break and 100% germination after 7 and 12 days.

***In vitro* shooting regenerative ability**

The regeneratedshootlets presented in shootlets number, elongation and number of leaves per shootlet formed of Brunfelsia were visibly enhanced by the lowest concentrations of sodium alginate (1%) and calcium chloride (2.5 and 5%) (A1, A2), that recorded the maximum shootlets number (3.67 up to 4.67 shootlet / explant) when conserved for various periods as (3, 5, and 7 weeks), also gave the highest numbers of leaves (17.67 and 22.67 leaf / shootlet) for 3weeks as compared to control. While, extending the period of conservation had significantly effected on multiplication rate of shoots that caused gradual reduction as shown in table 4 and fig.4. **The longest shootlets** (5.5 to 5.83 cm) were resulted from control treatment (3 and 5 weeks)

followed by sodium alginate (1%) and calcium chloride (5%) (3 weeks) which recorded 4.13cm as compared to other treatments that recorded shortest shootlets (Table 4). Most of synseed-derived plantlets were successfully acclimatized (fig.4, j) .This study is agreed with Singh and Gurung (2011) who indicated that envelope and non-encapsulated microshoot germinate after 2weeks. While the preservation period exceeded 25 days, encapsulation significantly decreased the number of shootlet and decline in plant recovery from conserved enveloped segments may be due to both deficiencies in the alginate beads and it speed drying. Fathy and Abd El-Kader (2012) on *Balanites aegyptiaca* plant noticed that 2g/l of sodium alginate gave the best number of shootlet and longhest shootlet after recovery. While, the leaves number formed per shootlet showed raising to 5.10 leaves when shootlet treated with sodium alginate at 3g /l. On *Hypochaeris radicata* experimented various levels of sodium alginate at 1, 2, 3, 4, 5, and 6% (w/v) and 1.016g of calcium chloride for 2, 4, and 6 months of storage period. They indicated that the highest rate of shoot proliferation in all explant segments obtained after four months. While the long storage of 6 months period has significantly reduced the regeneration ability of explants according to Jamuna and Paulsamy (2014). Sakr *et al*. (2018) on *Odontonema cuspidatum* mentioned that interaction between calcium chloride at (1.1g/100 ml) and sodium alginate 5% after 8weeks in capsules coats recorded the highest inhibition for shoot growth.

RAPD analysis of *in vitro Brunfelsia pauciflora* plantlets after conservation

In the present study, the genetic diversity among the preserved treatments such as (1) 1% Sodium alginate + 2.5g Calcium chloride/100ml distilled water; (2)1% Sodium alginate + 5g Calcium chloride/100ml distilled water; (3) 1% Sodium alginate +10g Calcium chloride/100ml distilled water; (4) 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water; (5) 3% Sodium alginate + 5g Calcium chloride/100ml distilled water and (6) Control plantlets (non-capsulated) and conserved for 7 weeks) was investigated using six RAPD primers, generating discernible reproducible banding profiles. (Fig.5) illustrates the RAPD profiles amplified with six of these primers. As illustrated in Table 5 the RAPD profiles amplified by the different primers comprised multiple bands ranging from 5 to 18 amplicons. The total number of DNA fragments amplified using six primers was an average of 9.5amplicons per primer. The number of polymorphic amplicons ranged from 2 to 15 with an average of 6.7 amplicons /primer. The monomorphic amplicons ranged from 2 to 3 with an average of 2.8/ primer. Primer OP- A-13 amplified the highest number of amplicons (18), and demonstrated the highest polymorphism percentage among the preserved treatments. While, the lowest number of amplicons was revealed by the primer OP-C-12. Therefore, the different primers expressed different levels of polymorphism, ranging from 40% with the primer OP-C 12 to 83.3% with the primer OP-A 13 with an average of 65.7%. The size of amplified fragments is diverse with various primers, ranging from 1686 to 209bp (Fig.5).

Genetic relationships and cluster analysis of the preserved culture as revealed using RAPD markers: To define the genetic relationships among the conserved treatments, the scoring data (1 for presence and 0 or the absence) resulting from the six tested primers were used to compute the similarity matrices according to Nei &Li's coefficient. These similarity matrices were used for generating a dendogram using UPGMA method. As shown in Table 6 the genetic similarity ranged from 56.1% to 80%. The highest genetic similarity revealed by the PAPD analysis (80%) was among (5) and (6), while the lowest (56.1%) was between (1) and (5).

The Nei & Li's RAPD – depend on coefficient of genetic resemblance between the conserved cultures was used to develop a dendrogram by UPGMA method (Fig.6). The dendrogram grouped the treatments of the preserved culture into two main clusters. The first cluster included the conserved cultures 1 and 2,while the second cluster was divivded into two subclusters,the frist included the preserved culture (3),and the other subcluster was subdivided into two groups the first inculuded (4) and the second group included (5) and (6). Therefore, the RAPD-based dendrogram grouped the nine wheat parental genotypes in complete accordance to their taxonomic classification. From these reults, It could be emphasized that molecular markers are potentially more efficient in taxonomic studies. These results were confirmed by a previous study on genetic stabilization of cultures obtained from syndetic seeds of woody species plants (Thendral Hepsibha *et al*., 2010), demonstrated the genetic distances between six species (Loc 1 to and Lco 6) of *Azima tetracantha* (Lam) using Nei'coefficient and dendrogram to the analysis cluster. They found that the lower similarity value of 0.085 indicates high diversity between Loc 1 and Loc 6 species. The genetic similarity index between Loc 2 and Loc 4 was highest with a value of 0.900. Aldoori *et al*. (2019) showed the differentiation between *in vivo* and *in vitro pongamia pinnata* using RAPD- DNA markers. They found that RAPD-PCR formed a total of 122 amplification fragments and genetic polymorphism was 39.34%. The results indicated low genetic polymorphism

were observed between the mother plant and the other two tissues culture treatment, the values was 39.34% using (RAPD) with high similarities ranged from 60.66 to 71.62%. The results indicated that there was high genetic similarity between the plants produced from tissue culture and mother plants. Faisal *et al*. (2012) found that the genetic stability of *Rauvolfia serpentine* cultures that were produced from encapsulation and conserved for four weeks was estimated by random amplified polymorphic DNA-RAPD markers. All established plantlets were monomorphic and conformable to the mother plant. Kovalchuk *et al*. (2008) indicated that introductory analysis found no significant differences among apple gemplasm plantlets that conserved for 39 months and control plant. Both RAPD and ISSR are characterized by simplicity, cost-effectiveness and success, so used to test the genetic stability of the artificial seeds that were regenerated from varying plants species Martins *et al*. (2004).

Anatomical structure

The anatomical sections made in *Brunfelsia paucifolra* leaf blade after two months of recovery (seven weeks of conservation) are shown in Fig. (7, 8) and Table 7. The leaf blade of the plant kept in control treatment has uniseriate upper and lower epidermis, with thin cuticle layers. The lower epidermis has a few number of stomata compared to the upper epidermis. The mesophyll tissue is made up of two distinct layers; palisade and spongy tissues. The palisade tissue is made of one elongated, rectangular parenchyma cells containing many chloroplasts followed by the loosely irregular cells which clustered together to form air spaces. The main vascular bundle is biocollateral with crescent shaped. Data contributed to the influence of low concentration of sodium alginate (1%) with different concentrations of calcium chloride ($CaCl_2.2H_2O$) on *Brunfelsia paucifolra* leaf blade are present in Fig. (7, 8) and Table 7. As compared to control, it is clear that increasing ($CaCl_2.2H_2O$) levels resulted in outstanding reductions in average midrib thickness, vascular bundle dimensions (length and width), and xylem tissue thickness. The calculated reductions percentages in the leaf midrib thickness were 3.87, 30.70, and 49.13% for A1, A2, and A3, respectively. The xylem vessels and palisade tissue cells appear wider in A1 treatment compared with all treatments. Relative to control, the increase percentage in xylem vessel diameter was 8.30%. A1 and A2 treatments caused a remarkable increase in leaf lamina, mesophyll tissue as well as palisade and spongy tissue thickness. Besides, the increase of percentages in leaf lamina thickness due to these treatments were; 22.63 and 9.01%, respectively. The increment in leaf lamina in these treatments as a result of the increase in thickness of palisade and spongy tissues also,large intercellular spaces are present in these tissues**.** In contrast, A3 treatment gives considerable thinner lamina (168.75 µ), more compacted leaf blade, reduced mesophyll tissue thickness as well as palisade tissue cells and number of spongy tissue rows. These results are in harmony with Hura (2017) on the effect of $CaCl_2$ in palisade tissue. The reductions in leaf measurements in this treatment may be explaining the reduction of explant ability to survive comparing with other treatments. The mesophyll tissue in A1 and A2 treatments is characterized by large size of palisade tissue cells with high density plastids and spongy tissue with large air spaces. The high survive ability with these treatments may be due to the increments that occurred in palisade tissue thickness with its dense plastids which have a role in photosynthesis process. Moreover, the present of large air spaces in the spongy tissue have a role in gas exchanges. In addition to, closed stomata and the ability of plants to store harmful salts in crystals form (A1). Concerning the effect of high concentration of sodium alginate (3%) with different concentrations of calcium chloride (CaCl2.2H2O); 2.5 and 5% on *Brunfelsia* leaf blade are given in Table 7 and Fig.(7, 8). It is noticed that C2 treatment showed a relatively remarkable increase in leaf anatomical features compared with control. The increments compared with control were; 26.73, 4.38, 9.34, and 47.90 % for the vascular bundle length, xylem tissue, phloem tissue thickness, and xylem vessel diameter, respectively. In addition to, C2 and C3 treatments induced considerable increases in leaf lamina thickness and its internal tissues. These treatments showed a prominent increase in number of trichomes which related to protection of plants from damaging and give resistant to external stress (Zhang *et al*. 2020).

Conclusion

From these results, the study demonstrated that the method described in this work may be potentially used to conserve eligible *Brunfelsia* plants for a short period. This could also facilitate the transport of encapsulated explants among laboratories and extension centers of distant places. For the large- scale application of process, further experiments are needed to achieve a higher percentage of conservation after the storage of encapsulation explants of *Brunfelsia*.

Declarations

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Author contributions TAA and LST supervised and revision the whole work, SAS carry out section the anatomical structure of manuscript. M H M helped in the work. A M M G performed genetic stability assessment using RAPD-DNA analysis. A I A R A performed the experiments, analyzed the data and wrote the manuscript. Thanks to all authors that take part to complete this work.

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Data availability/strong>undefineddatasets generated or analyzed during this study are included in this article.

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Tables

Table 1 Sequance of 18 decamer arbitrary primers assayed in RAPD – analysis to detect polymorphism among nine wheat parental genotypes

Primer code	Sequence (5′-3′)
OP-A 10	5´- GTG ATC GCA G - 3 ´
OP-A 13	5´ - CAG CAC CCA C - 3´
OP-C 12	5´- TGT CAT CCC C - 3´
OP-D 07	5 ´ TTG GCA CGG G - 3´
OP-M 09	5´- GTC TTG CGG A - 3´
OP-Y-09	5´ - AGC AGC GCA C - 3´

Table 2 Effect of cytokinin types on *in vitro s*hootlet propagation of *Brunfelsia Pauciflora* plant during three repeated subcultures

Parameters Treatments		Subculture 1			Subculture 2			Subculture3		
BAP (mg/l)	Kin (mg/l)	No. shootlet /Explant	Length Shootlet (cm)	No. leaves/ Explant	No. shootlet/ Explant	Length Shootlet (cm)	No. leaves/ Explant	No. shootlet/ Explant	Length Shootlet (cm)	No. leaves/ Explant
Control (MS. free)		1.00^{d}	2.16^{a}	11.33^{d}	2.00^{d}	1.76^{ab}	11.00^{d}	2.33^{b}	1.16^{ab}	13.00^{d}
1	0	6.33^{bc}	0.83^{bc}	25.33^{b}	10.00^{bc}	1.5^{abc}	27.67^{b}	3.33^{b}	0.67^{b}	16.67^{cd}
2	0	14.67^{a}	1.16^{b}	37.33^{a}	16.00^{a}	0.83^{abc}	42.33^{a}	11.67^{a}	1.83^{a}	39.00^{a}
1	1	4.00^{cd}	0.63^{c}	18.33^{c}	8.00^{c}	1.13^{abc}	29.00^{b}	5.00^{b}	0.5^{b}	22.33^{c}
1	2	4.33^{c}	0.50^{c}	13.33^{cd}	6.67^{cd}	0.43^{c}	15.00^{cd}	5.00^{b}	0.67^{b}	16.00^{cd}
2	1	8.00^{b}	0.67^{bc}	26.33^{b}	14.00^{ab}	0.73^{bc}	44.33^{a}	10.33^{a}	1.67^{ab}	28.67^{b}
2	2	9.00^{b}	0.60^{bc}	25.00^{b}	8.00^{c}	2.00^{a}	19.67^{c}	5.00^{b}	0.40^{b}	22.00^{c}

Means in a column with similar letters are not significantly different according to Duncan's Multiple Range Test (DMRT)at 0.05% level.

Table 3 Optimization of micropropagated *Brunfelsia paucifolra* under effect of different plant growth regulator

Concentrations of growth regulators per (mg/l)				Parameters					
2ip	BAP	IBA	NAA	No.of shootlet/ explant	Length shootlet (cm)	NO. of leaves/ Shootlet	Rooting (%)	NO. Roots/ Shootlet	Length of Root (cm)
0	0	0	0	4.33^{abc}	2.5^{c}	18.33^{abcd}	0	0	0
1	0	0	0	4.33^{abc}	3.00^{c}	28.33^{a}	0	0	0
1	2	0.4	0	6.33^{a}	2.50^{c}	20.67^{abcd}	0	0	0
1	2	0.8	0	6.67^{a}	3.70^{bc}	21.33^{abc}	0	0	0
1	0	0	2	3.00^{abc}	3.33^{bc}	23.00^{abc}	33.33^{b}	0.33^{b}	0.20^{b}
1	0	0	4	2.00^{bc}	3.17^{c}	14.00^{bcd}	0	0	0
1	2	0	2	3.33^{abc}	4.17^{bc}	18.33^{abcd}	33.33^{b}	0.33^{b}	0.33^{b}
2	0	0	0	4.33^{bc}	9.00^{a}	30.33^{a}	33.33^{b}	1.33^{ab}	1.50^{b}
2	2	0.4	0	5.33^{ab}	3.77^{bc}	18.33^{abcd}	33.33^{b}	0.67^{b}	1b
2	2	0.8	0	3.00^{abc}	4.33^{bc}	10.67^{cd}	33.33^{b}	0.67^{b}	1.33^{b}
2	2	0	2	1.00^{c}	5.50^{b}	26.33^{ab}	100^{a}	2.67^{a}	6.26^{a}
2	0	0	2	4.33^{abc}	3.00^{c}	19.00^{abcd}	0	0	0
2	0	0	4	1.67^{bc}	3.33^{bc}	9.33^{d}	33.33^{b}	0.33^{b}	1.67^{b}

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05%

Table 4 Influence of various concentrations of sodium alginate and calcium chloride during different conservation periods on shooting regenerative ability of *Brunfelsia paucifolra*

parameters	Number of shootlet /explant			Number of leaves/explant			Length of Shootlet (cm)		
Treatments	3week	5week	7week	3week	5week	7week	3week	5week	7week
Control	2.00^{c}	2.00^{a-c}	2.00^{a-d}	10.33^{b}	11.33^{a}	11.33^{a}	5.5^{a}	5.83^{a}	2.76^{a}
A1	4.67^{a}	3.67^{a}	3.00^{ab}	17.67^{a}	7.33^{a-c}	5.33^{bc}	1.03^{c}	0.76^{bc}	1.1^{a}
A2	4.67^{a}	4.33^{a}	4.00^{a}	22.67^{a}	8.67^{ab}	8.00^{ab}	4.13^{b}	1.36^{b}	1.16^{a}
A3	2.33^{bc}	2.00^{bc}	0.33^{cd}	11.00^{b}	4.00^{c-e}	0.33^{d}	0.43^{cd}	0.56^{c}	0.1^{b}
B1	3.00^{a-c}	2.00^{bc}	1.00^{bcd}	5.67^{bc}	1.67^{de}	1.33^{cd}	0.36^{cd}	0.16^{c}	1.00^{a}
B2	4.33^{a}	3.00^{ab}	2.00^{a-d}	6.33^{bc}	4.33^{c-e}	4.00^{bcd}	1.17^{c}	0.43^{c}	1.16^{a}
B3	0.00	0.00	0.00	00.00	0.00	0.00	0.00	0.00	0.00
C1	4.00^{ab}	3.33^{ab}	1.00^{bcd}	6.00^{bc}	4.67^{bcd}	2.00^{cd}	0.53^{cd}	0.2^{c}	0.8^{ab}
C2	3.33^{a-c}	3.00^{ab}	2.33^{a-c}	6.67^{bc}	3.67^{de}	3.00^{cd}	0.83^{cd}	0.47^{c}	0.83^{ab}
C3	0.00	0.00	0.00	00.00	0.00	0.00	0.00	0.00	0.00

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test at 0.05% level

Table 5 Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by RAPD markers among the preserved treatments

Primer code	Total no. of amplicons	Monomorphic amplicons	Polymorphic amplicons	Polymorphism %
OP-A 10	8	2	6	75.0
OP-A 13	18	3	15	83.3
OP-C 12	5	3	2	40.0
OP-D 07	9	3	6	66.7
OP-M 09	8	3	5	62.5
OP-Y-09	9	3	6	66.7
Total	57	17	40	
Average	9.5	2.8	6.7	65.7

Table 6 Genetic similarity matrix computed according to Nei & Li's from RAPD data for the conserved culture

Treatments	1	2	3	4	5	6
1	100					
2	72.4	100				
3	68.9	70.1	100			
4	58.6	67.7	70.8	100		
5	56.1	68.9	75	75.4	100	
6	58.1	66.7	69.6	72.7	80.0	100

Table 7 Microscopic measurements (μ) and counts of certain anatomical features in cross sections of *Brunfelsia pauciflora* leaf blade after recovery (seven weeks of conservation) at the stage of two months (Average of 3 readings)

Treatments	Con.	A1	% to con.	A2	%to con.	A3	% to con.	C1	% to con.	C2	% to con.
Haracteristics											
Mid rib thick.	565.25	543.38	-3.87	391.72	-30.70	287.50	-49.13	512.10	-9.40	581.88	2.94
Vascular bundle length.	147.95	112.80	-23.75	141.83	-4.13	125.00	-15.51	145.99	-1.32	187.50	26.73
Vascular bundle width.	326.97	323.55	-0.13	203.52	-37.76	187.50	-42.66	297.95	-8.87	287.50	-12.07
Xylem tissue thick.	78.39	41.36	-47.24	64.76	-17.39	36.36	-53.61	65.53	-16.40	81.82	4.38
xylem vessel diameter	12.17	13.18	8.30	12.27	0.82	6.25	-48.64	8.96	-26.37	13.00	6.82
Phloem tissue thick.	53.86	56.65	5.12	40.80	-24.25	18.75	-65.19	41.26	-23.39	58.89	9.34
Lamina thick.	210.11	229.05	9.01	257.66	22.63	168.75	-19.68	281.42	33.94	261.54	24.48
Mesophyll tissue thick.	155.71	189.91	21.96	222.56	42.93	125.00	-19.72	232.82	49.52	223.08	43.27
Palisade tissue thick.	29.36	46.30	57.70	51.72	76.16	34.38	17.09	38.29	30.42	42.31	44.11
Spongy tissue thick.	118.49	143.62	21.21	174.48	47.25	87.50	-26.15	208.13	75.65	192.31	62.30
Upper epidermis thick.	32.94	19.46	-40.92	21.76	-33.94	18.75	-43.08	27.79	-15.63	26.92	-18.27
Lower epidermis thick.	31.52	31.07	-1.43	21.12	-32.99	12.50	-60.34	20.03	-36.45	19.23	-38.99

Figures

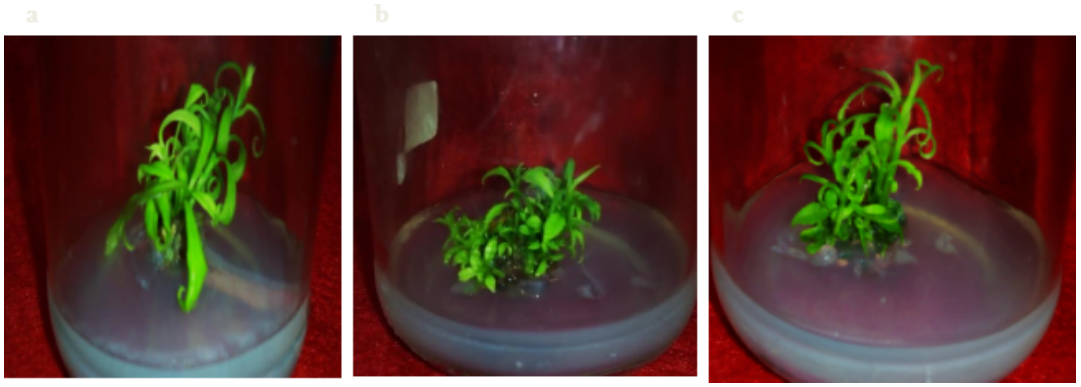

Figure 1

In vitro shootlet proliferation of *Brunfelsia pauciflora* plant effecting by MSculture medium supplemented with various cytokinins types : (a): Control (MSfree hormones), (b): MS plus BAP (2mg/l) and (c): MS+ BAP (2mg/l) plus Kin (2mg/l)

Figure 2

Optimization of micropropagated *Brunfelsia paucifolra* under effect of different plant growth regulator (PGR): (a): Control (MS free hormones), (e): MS+2ip (2mg/l), (f): MS+2ip (2mg/l)+ BAP (2mg/l)+ NAA(2mg/l)

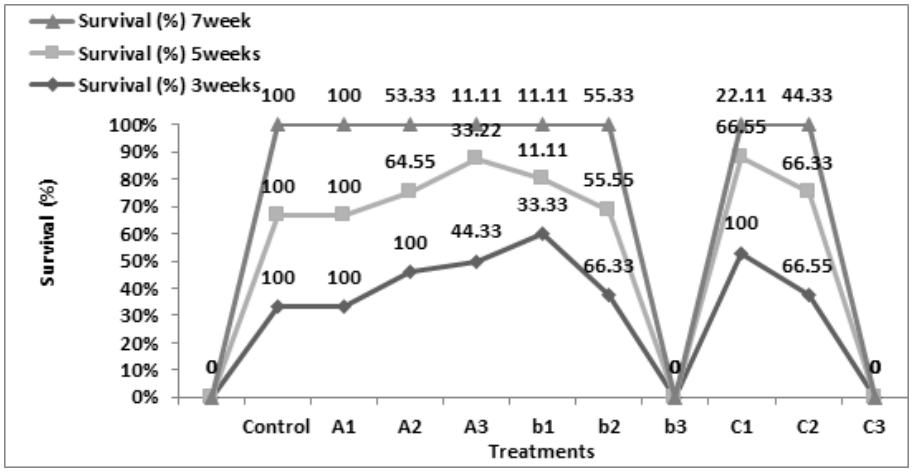

Figure 3

Effect of different varied concentrations of sodium alginate and calcium chloride on regrowth ability of encapsulated explants of *Brunfelsia Paucifolra* for various conservations periods

Figure 4

Sprouting and development of *in vitro* capsulated explants: (g) conservation of *Brunfelsia paucifolra* synseeds at room teperature ; (h) different sprouting stages of synseeds; (i) early phase of *in vitro* shoot formation and elongation; (j) synseed-derived acclimatized plantlets

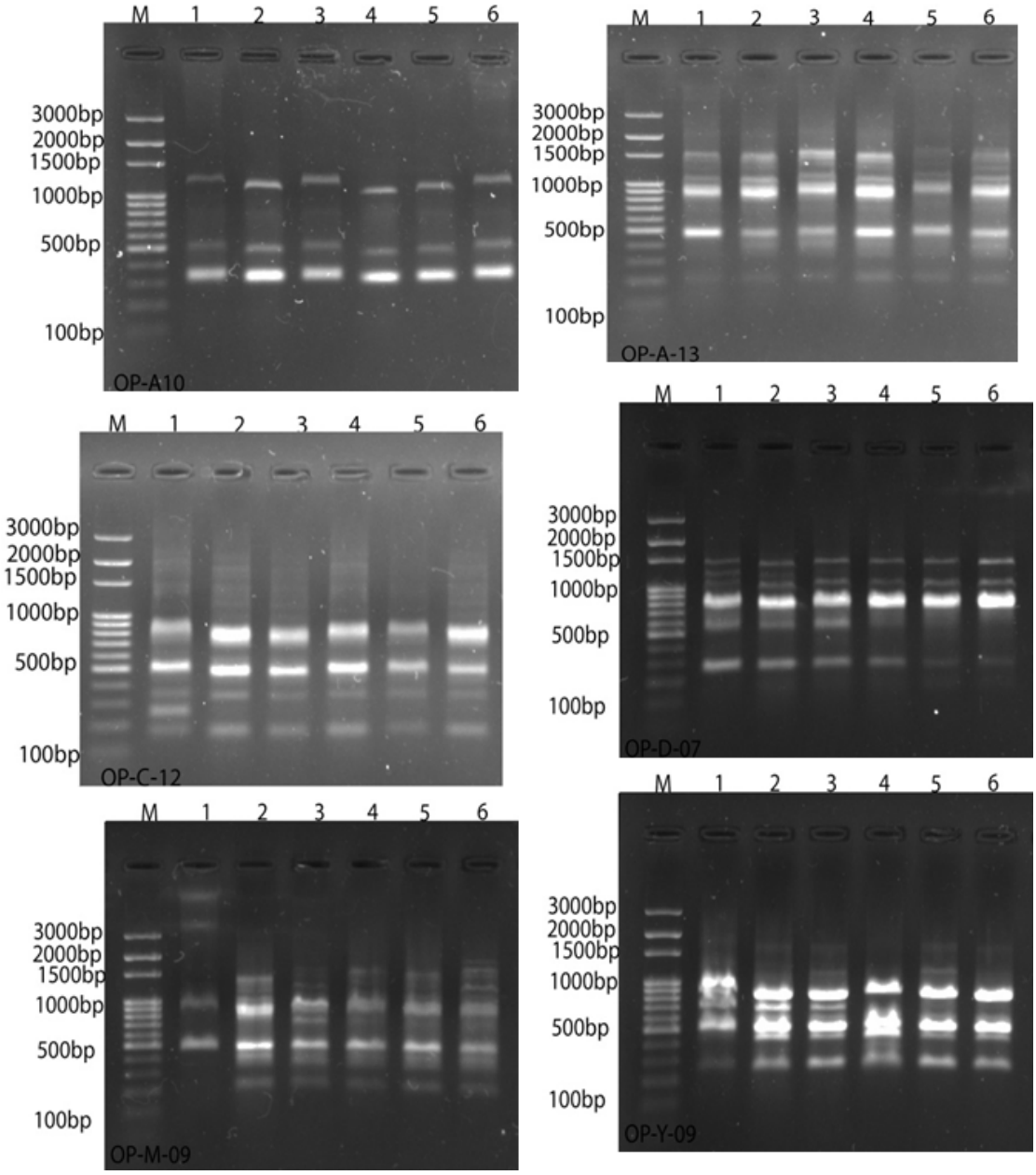

Figure 5

RAPD pattern of the *in vitro* conservation treatments of *Brunfelsia pauciflora* with 6 primers: OP-A-10, OP-A-13, OP-C-12, OP-D-07, OP-M-09 and OP-Y-09, M. DNA marker. (1): 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water; (2):1% Sodium alginate + 5g Calcium chloride/100ml distilled water;(3): 1% Sodium alignate+10g Calcium chloride/100ml distilled water; (4): 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water; (5): 3% Sodium alginate + 5g Calcium chloride/100ml distilled water and (6): Control plantlets (non-capsulated) and conserved for 7 weeks. M= 100 bp marker (solis biodyne)

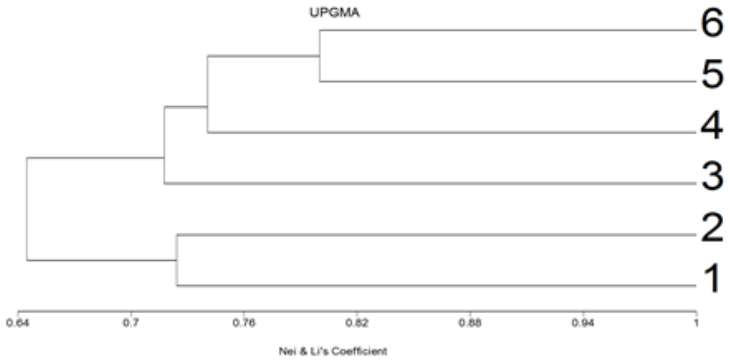

Figure 6

Dendrograms showing similarity matrices for the preserved cultures i.e.(1): 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water; (2):1% Sodium alginate + 5g Calcium chloride/100ml distilled water; (3): 1% Sodium alignate+10g Calcium chloride/100ml distilled water; (4): 1%Sodium alginate + 2.5 g Calcium chloride/100ml distilled water; (5): 3% Sodium alginate + 5g Calcium chloride/100ml distilled water and (6): Control plantlets (non-capsulated) and conserved for 7 weeks. using Nei&Li's similarity coefficients based on RAPD markers (UPGMA)

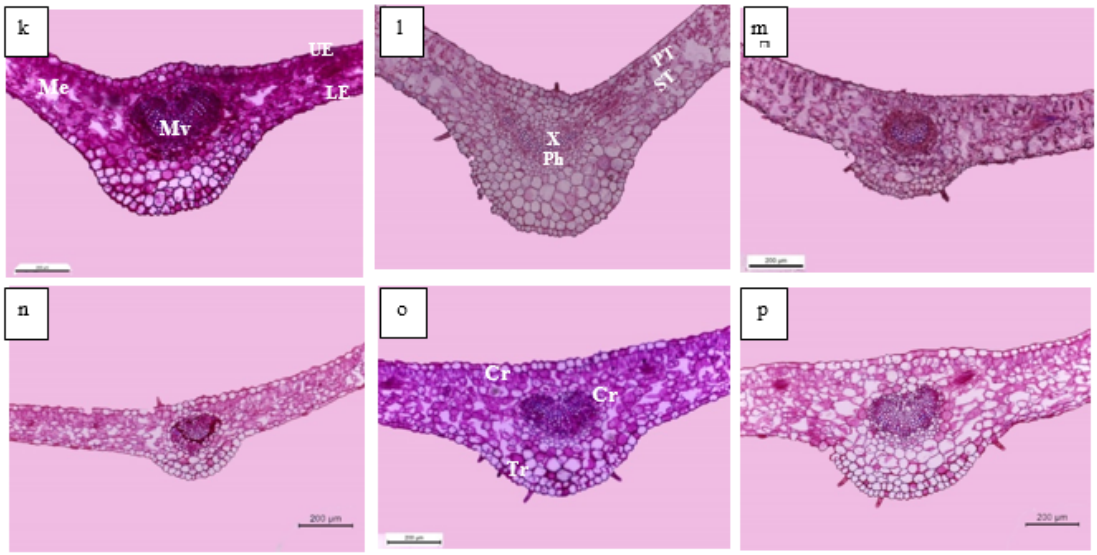

Figure 7

Photomicrographs of transverse sections of *Brunfelsia pauciflora* leaf blade showing anatomical changes after recovery. k): Control ; l): A1, Sodium alginate 1% + Calcium chloride 2.5%, m): A2, Sodium alginate 1% + Calcium chloride 5%, n): A3, Sodium alginate 1% + Calcium chloride 10% , o) Sodium alginate 3% + Calcium chloride 2.5%, p) Sodium alginate 3% + Calcium chloride 5%, CU, Cuticle; UE, Upper epidermis; LE, Lower epidermis; Mv, Main vascular bundle; Me, Mesophyll; PT, Palisade tissue; ST, Spongy tissue; X, Xylem, Ph, Phloem; Cr, Crystal; Tr, Trichome

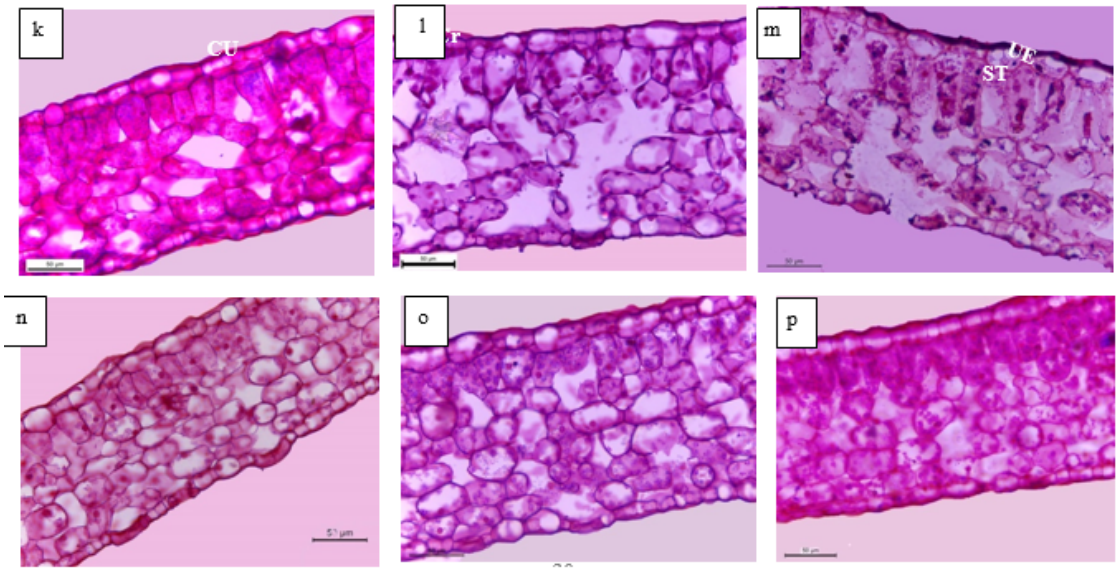

Figure 8

Photomicrographs of transverse sections of *Brunfelsia pauciflora* leaf lamina showing anatomical changes after recovery (seven weeks of conservation) at the age of two months. k): Control ; l): A1, Sodium alginate 1% + Calcium chloride 2.5%, m): A2, Sodium alginate 1% + Calcium chloride 5% n): A3, Sodium alginate 1% + Calcium chloride 10% , o): Sodium alginate 3% + Calcium chloride 2.5%, P:) Sodium alginate 3% + Calcium chloride 5%, CU, Cuticle; UE, Upper epidermis; LE, Lower epidermis; Me, Mesophyll; PT, Palisade tissue; ST. Spongy tissue; Cr, Crystal