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Regeneration of *Parkia biglobosa* Benth.: An important tree species of Africa

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ABSTRACT

Parkia biglobosa Benth is a perennial deciduous tree which has many uses in Nigeria. The population of the tree is rapidly declining, but there are no conservation efforts to prevent it from extinction. To conserve this genetic resource, tissue culture studies were carried out using seeds collected from three locations (Boki, Obanliku and Obudu). Leaf and hypocotyl explants from in vitro germinated seedlings were cultured on MS medium containing different concentrations (1, 1.5 and 2 mg l^{-1}) 6-benzylaminopurine (BA) either alone or in combination with 0.2 mg l^{-1} 1-naphthaleneacetic acid (NAA) for callus induction. The medium supplemented with 1 mg l^{-1} BA and 0.2 mg l^{-1} NAA was the optimum growth regulator combination for callus induction in both explants. Seeds from Boki produced the highest percentage (80%) of callus from leaf explants, while those from Obanliku recorded the highest (55.4%) from hypocotyl explants. Shoots were regenerated from callus initiated from hypocotyl explants, but not from leaf explants upon transfer to MS medium supplemented with 0.2 mg l^{-1} BA either alone or in combination with 0.2 mg l^{-1} NAA. The highest regeneration frequency (39.4%) was observed for Obanliku accession on MS medium supplemented with BA alone. Of the regenerated shoots, 31.6% rooted on MS medium containing 0.2 mg l^{-1} Indole-3-acetic acid (IAA). Seventy percent of the regenerated shoots were diploid by flow cytometric analysis. This protocol will be useful not only for vegetative propagation but also for plant genetic transformation and gene function studies of P. biglobosa.

Key words: *Parkia biglobosa* Benth. Regeneration. Organogenesis. Trees. Ploidy.

INTRODUCTION

In Nigeria, several tree species serve as sources of wood, food, fodder and medicine to indigenous people. The trees also provide ecological services including soil fertility and microclimate amelioration. In addition to direct domestic use of the tree products, they are a source of cash for many poor people. However, due to continuous allocation of the existing forest to urban, agricultural, recreational and other uses, the forest is being depleted and many of these important trees are gradually becoming extinct [1]. To prevent extinction and derive

maximum benefits from the indigenous plants, it is necessary to preserve the germplasm. Vegetative propagation by means of tissue culture techniques is an important tool for plant germplasm conservation and rapid clonal multiplication as well as for reforestation and tree improvement [2-4].

Parkia biglobosa popularly known as the African locust bean tree belongs to the family Fabaceae [5, 6]. It is a perennial deciduous tree occurring in a belt between 5° N and 15° N [6], 7 to 20 m tall, and in some cases it can reach up to 30 m [7]. The fruit is a slightly curved, brown indehiscent pod, 30 to 40 cm long and 2 to 3 cm wide producing up to 20 seeds. The seeds when boiled and fermented is known as 'dawadawa' in Hausa language in Nigeria, a black strong smelling tasty seasoning, rich in lipid 29 per cent, protein 35 per cent, carbohydrate 16 per cent, and it is a good source of fat and calcium for rural dwellers [8]. The pods are used as sponges and strings, dyes, and for fishing, and also for preparing insecticide powder [6]. The bark is used as a mouthwash, vapour inhalant for toothache, or for ear complaints. It is macerated in baths for leprosy and used for bronchitis, pneumonia, skin infections, sores, ulcers, and washes for fever, malaria, diarrhoea, and sterility. Roots are used in a lotion for sore eyes."

In spite of these economic values of *Parkia*, the population of the plant is declining at an alarming rate, and there are no conservation measures. One reason for this decline is uncontrolled bush burning through farming, which is a common feature in Nigeria during the dry season. Bush burning destroys the young seedlings and reduces the population density. Another factor is excessive grazing by cattle and other domestic stock. These animals not only eat up the young and tender seedlings, but may also physically destroy them during movement within the vegetation. A more important factor is the fact that only a small percentage of the seeds germinate in the field, a lot more are dormant [9].

To prevent *P. biglobosa*, from becoming extinct, there is need to conserve germplasm through tissue culture. Tissue culture studies in *Parkia biglobosa* Benth are scanty. Existing literature is confined to callus and embryo induction without shoot or plantlet regeneration [10] or in vitro germination and multiplication of a Senegalese *P.biglobosa* [11]. Plant regeneration has been successful in some non-woody horticultural and woody plants [12-17]. The present report, therefore, describes for the first time a method for differentiation of callus, shoots and plant regeneration from hypocotyl explants of *Parkia biglobosa* Benth via indirect organogenesis. It is our expectation that adoption of this protocol will allow the regeneration and transformation studies of *P. biglobosa* in laboratories where it is needed, as well as provides an opportunity to extend and improve the current protocol for further improvement of the economic traits of this plant.

MATERIALS AND METHODS

Plant material and seed germination

Matured seeds of *Parkia biglobosa* Benth were collected from three locations in northern Cross River state (Indi-abeb in Obudu Local Government Area, Bafin in Boki Local Government Area, and Sankwala in Obanliku Local Government Area), Nigeria. In this study, the locations are referred to as accessions. The seeds were chemically scarified using 70% H_2SO_4 for 20 min; surface sterilized by treating with 1% ($^{v}/_{v}$) sodium hypochlorite for 15 min, and rinsed three times with sterile distilled water. Seeds were then germinated on MS [18] medium, with 10 g/l sucrose and 8 g/l agar (Wako Pure Chemical Industry Ltd, Japan) in a growth room at 25±1℃, 16 h photoperiod, 30-40µmol m^{-2} s^{-1} cool white fluorescent light. The pH of the medium was adjusted to 5.8 prior to the addition of agar and autoclaving at 121℃ for 15min.

Callus induction

For callus induction, six types of basal media were used as shown in Table 1. Hypocotyls and leaves about 5 mm long were excised from 2 week-old seedlings and cultured in Petri-dishes containing 33 ml of callus induction medium. For each medium type, 3 replications per location (accession), each replication with 2 Petri-dishes, and each Petri-dish contained 10-15 explants of a particular accession. An average of 360 explants (hypocotyls and leaves) per cultivar was used in the experiment. All cultures were placed at 25±1℃, 16 h photoperiod, 30-40µmol m^{-2} s^{-1} cool white fluorescent light. The explants were subcultured at two weeks intervals until callus developed.

Regeneration of plants from callus

Calli were transferred to MS medium containing 0 .2 mg l^{-1} 6-benzylaminopurine (BA) alone (MBA) or in combination with 0.2 mg l^{-1} NAA (MBAN). Both media were supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} agar. For regeneration, calli were cultured in Petri-dishes containing these media for eight weeks in a growth room at 25±1℃, under 16 h photoperiod of 30-40µmol m^{-2} s^{-1} with cool white fluorescent light. After 8 weeks of culture on regeneration medium, healthy shoots of approximately 2-3 cm long were transferred to MS medium containing 0.2mg l^{-1} IAA for elongation and rooting.

Flow cytometry analysis

In vitro-grown leaves of regenerated plants and the control plants (germinated plants) were chopped with a razor blade into an ice cold nuclei isolation buffer (Cystein UV Precise P, Partec GMbH, Germany). The samples were filtered through a 30µm mesh and stained with 4, 6-diamidino-2-phenylindole (DAPI). Fluorescence intensity of isolated nuclei was measured using a Partec PA II ploidy analyzer equipped with an argon ion laser (488 nm). The flow rate did not exceed 30 fluorescent events per s in all cases and the fluorescence of at least 5,000 particles (counts) was recorded.

Statistical analysis

The experimental design was a completely randomized design with three replications per medium type, 10-15 explants per accession per replicate. For the experiment on callus induction, 3 accessions, 2 types of explants at 6 types of media were analyzed as a 3 x 2 x 6 factorial arrangement. In this study, callus from leaf explants did not regenerate shoots, therefore, the analysis on regeneration were based on the number of hypocotyls explants cultured. Hence, 3 accessions at 2 types of media were analyzed as a 3 x 2 factorial arrangement. Data obtained were subjected to analysis of variance (ANOVA). Significant means were separated using least significant difference (LSD) test. All percent data were subjected to arc sine (√x) transformation before statistical analysis.

RESULTS AND DISCUSSION

Seed germination and callus induction

P. biglobosa seeds started germinating after 7 days of sowing in vitro. After 2 weeks, the percentage of germination was relatively high ranging from 85% to 92% (data not shown). Seed dormancy has been reported to be a major problem in *P. biglobosa* [9]. Different methods to break dormancy in *P. biglobosa* seeds have been reported [9,19-20]. Our result indicates that the treatment used in this study is effective for breaking dormancy in *P. biglobosa* seeds.

Callus started to form 10 days after culture of leaf (Fig. 1a) and hypocotyl (Fig. 1b) explants in all the accessions and media used. Among the accessions used, Boki produced the highest

percentage (80%) of callus from leaf explants after four weeks of culture, while Obudu recorded the lowest (50.7%) (Fig. 2).On the other hand, the rate of callus formation from hypocotyl explants was higher (55.4%) in Obanliku and lower (34%) in Obudu (Fig. 2) . The variation in callus proliferation among the accessions indicates high genetic diversity within the species and that genotype maybe an important factor for in vitro regeneration of *P. biglobosa*. There were differences in the nature of calli produced from the different explants. Calli produced from leaf explants were white and more watery than those produced from hypocotyl explants, suggesting that callus morphology were influenced by the source of explant.

The effect of different media (T1-T6, Table 1)) on induction of callus is presented in Fig. 3. All the media induced callus at different rates. Of the different types of media used, the medium supplemented with 1 mg l^{-1} BA and 0.2 mg l^{-1} NAA (T4) proved to be the optimum growth regulator combination for callus induction in leaf and hypocotyl explants (Fig. 3). Generally, there was a low rate of callus proliferation from hypocotyl explants compared to leaf explants. Variation among the explants in the rate of callus induction maybe due to predisposition of tissues from some organs to more rapid cell division than others and the fact that even closely associated tissues from one organ have different potentials. The addition of 0.2 mg l^{-1} NAA in the media T5 and T6 caused apparent decrease in the rate of callus induction in hypocotyl explants. Comparing our results to those of [10], suggests considerable variation in response of explants to growth regulators. In Amoo and Ayisire, [10], when cotyledonary explants of *Parkia* were cultured in media supplemented with either 2,4-D or NAA, all treatments containing NAA did not produce callus. These discrepancies may reflect differences in genotypic response to regeneration.

Shoot regeneration from callus

Callus produced from the leaf and hypocotyl explants were transferred to MS medium containing 0 .2 mg l^{-1} 6-benzylaminopurine (BA) alone (MBA) or in combination with 0.2 mg l^{-1} NAA (MBAN). Callus from hypocotyl explants produced shoots with shoot clusters upon transfer to regeneration medium (Fig. 1c). On the contrary, callus from leaf explants did not produce shoots after several weeks of culture. This maybe as a result of inadequate combination of plant growth regulators or possibly shoot formation is restricted to hypocotyls explants in these accessions. Tidema and Hawker [21] reported callusing from leaf explants of three *Euphorbia* species (*E. peplus*, *E. lathyris* and *E. tannensis*), but shoot regeneration did not occur in many of the BA-NAA combinations that were studied. In this study, the highest regeneration frequency (39.4%) was observed on MS medium supplemented with BA alone (Fig.4). Irrespective of the medium, Obanliku accession produced significantly ($P>0.05$) more shoots than the other accessions. This result corresponds with the callus induction rate observed for the same accession, indicating that Obanliku accession is more responsive to tissue culture than the other accessions. The regenerative response of callus decreased significantly when 0.2 mg l^{-1} BA was supplanted with 0.2 mg l^{-1} NAA (MBAN). A synergistic effect of auxin and cytokinin was also reported in *Tamarindus indica* [22] and *Albizzia jebbeck* [23]. We, therefore, suggest that BA is efficient for callus induction and shoot regeneration in *P. biglobosa* using hypocotyls as explants. The same report was made by [24] in *Balanites aegyptiaca* and [25] in *Oroxylum indicum* (L.) Vent.

Shoots so inducted were transferred MS medium supplemented with 0.2 mg l^{-1} IAA for elongation and rooting. After 6 weeks of culture, some of the shoots reached between 7-9 cm in length (Fig. 1d), but without roots. Rather, callus proliferated at the base of the shoots. After a further subculture for 5 weeks, only 31.6% of the elongated shoots rooted (data not shown). This result suggests that IAA is probably not an efficient plant growth regulator for rooting in *P.biglobosa* or possibly the concentration used here is insignificant to have caused vigorous

rooting of the shoots. In contrast to our result, Jaiwal and Gulati [22] reported significant root formation in *Tamarindus indica* when shoots were cultured on MS medium supplemented with $5.7x10^{-6}$ M IAA, indicating that genotypic difference is a major factor in vitro regeneration. When Sambe et al. [11] cultured shoots derived from apex and cotyledons of a Senegalese species of *P. biglobosa* in IBA during their multiplication experiment, only 41.66% and 58.33% of the shoots, respectively, rooted. Although there maybe some genotypic differences between the Nigerian and the Senegalese *P. biglobosa*, comparing the two results indicates that irrespective of the explants and plant growth regulator used, rooting in *P. biglobosa* is rather difficult. Chèvre [26] opined that rooting is often more difficult at the ligneous plants than at the herbaceous plants. At high concentration of IBA (2 mg l^{-1}), Indieka et al. [13] reported that only 33% of the regenerated shoots rooted in *Melia volkensii*, confirming further that rooting is really difficult in tree species.

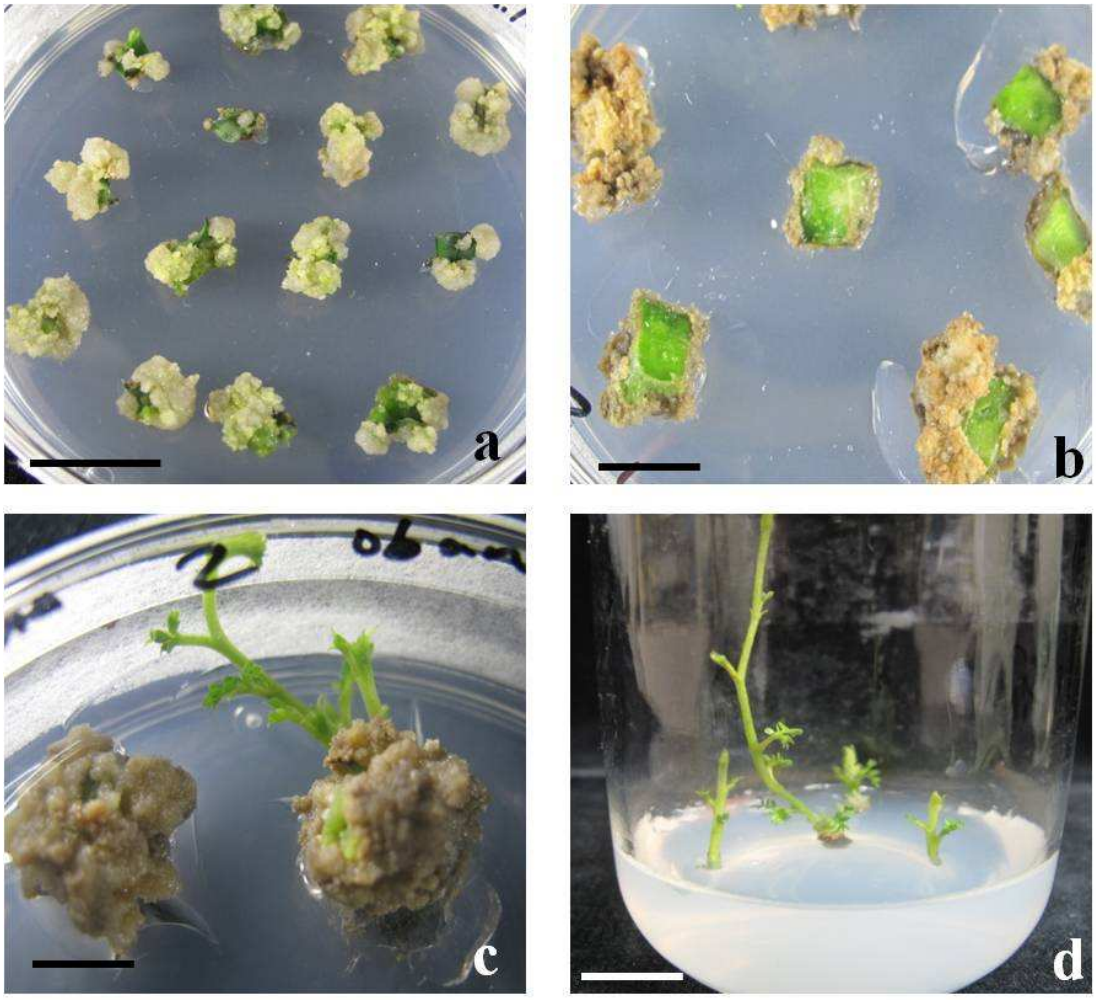

Fig. 1 Plant regeneration in *Parkia biglobosa* Benth. a Callus induction from leaf explants (*bar* = 1cm). b Callus induction from hypocotyl explants (*bar* = 1cm). c Shoot regeneration from callus inducted from hypocotyls explants (*bar* = 1cm). d Shoot elongation on MS medium supplemented with 0.2mg/l IAA (*bar* = 1cm)

Ploidy analysis

Since there were few rooted plantlets, all of them were used for ploidy studies by flow cytometry. Of the plants analyzed, 23.65, 29.45 and 32.7% in accession Obudu, Boki and Obanliku, respectively, were a mixture of mixoploids (2n+4n, 2n+8n) and tetraploids (4n) (Fig. 5 and 6). Although the percentage of polyploids recorded in our study is high, the regeneration of plants with high polyploidy has been reported earlier [17, 27]. Genetic instability is a regular phenomenon is plant tissue culture. Genetic instability such as polyploidy, aneuploidy, etc.

through callus cultures has also been reported in forest trees [28-30]. The genetic instability observed in this study may have been induced by prolonged exposure of cells to plant growth regulators in the culture medium. Detail cytological analyses are required to confirm these ploidy levels. However, the variations recorded here could be a useful tool for genetic improvement of this important woody species.

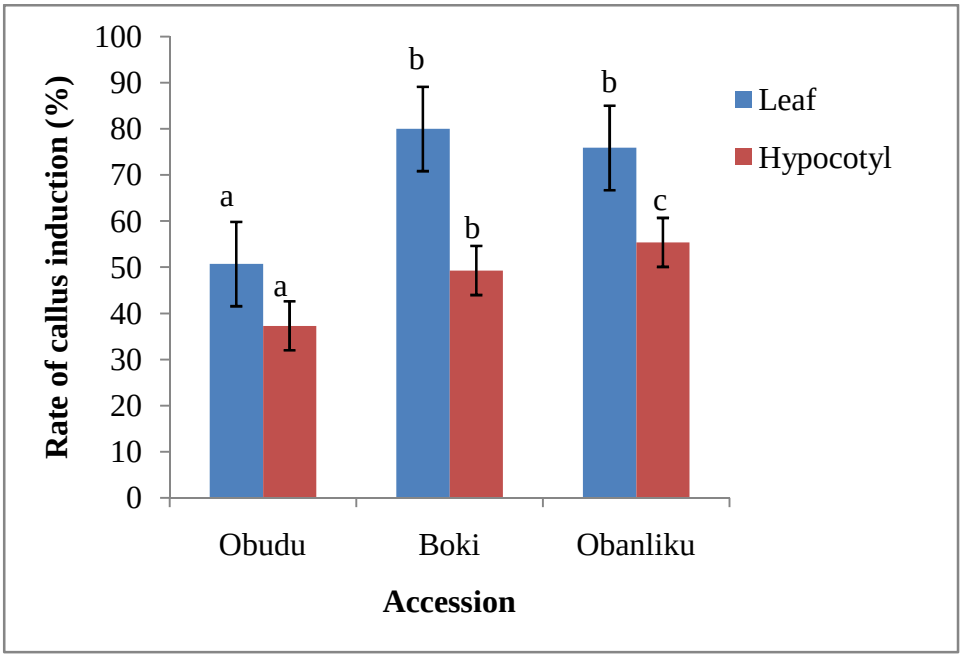

Fig 2 Rate of callus induction from leaf and hypocotyl explants of *Parkia biglobosa* Benth. Values with different case letters in a given accession are significantly different using least significant difference (LSD) test at 5% level

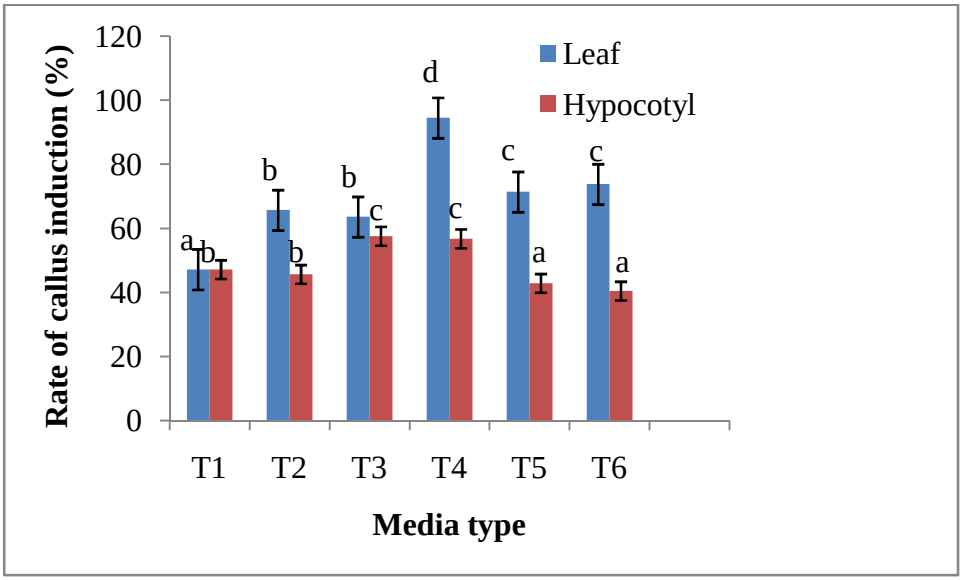

Fig.3 Effect of different media type on callus induction rate from leaf and hypocotyl explants of *Parkia biglobosa* Benth. Values with different case letters within an explant type are significantly different using least significant difference (LSD) test at 5% level

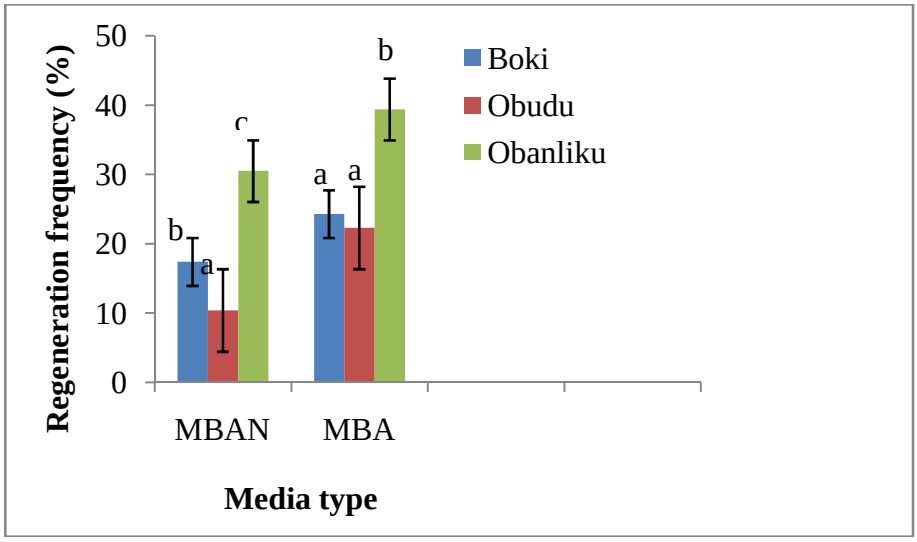

Fig. 4 Effect of plant growth regulators on shoot regeneration from hypocotyl-derived callus of *Parkia biglobosa* Benth. Calli were cultured on MS medium containing 0 .2 mg l^{-1} 6-benzylaminopurine (BA) alone (MBA) or in combination with 0.2 mg l^{-1} NAA (MBAN). Values with different case letters within a given medium type are significantly different using least significant difference (LSD) test at 5% level

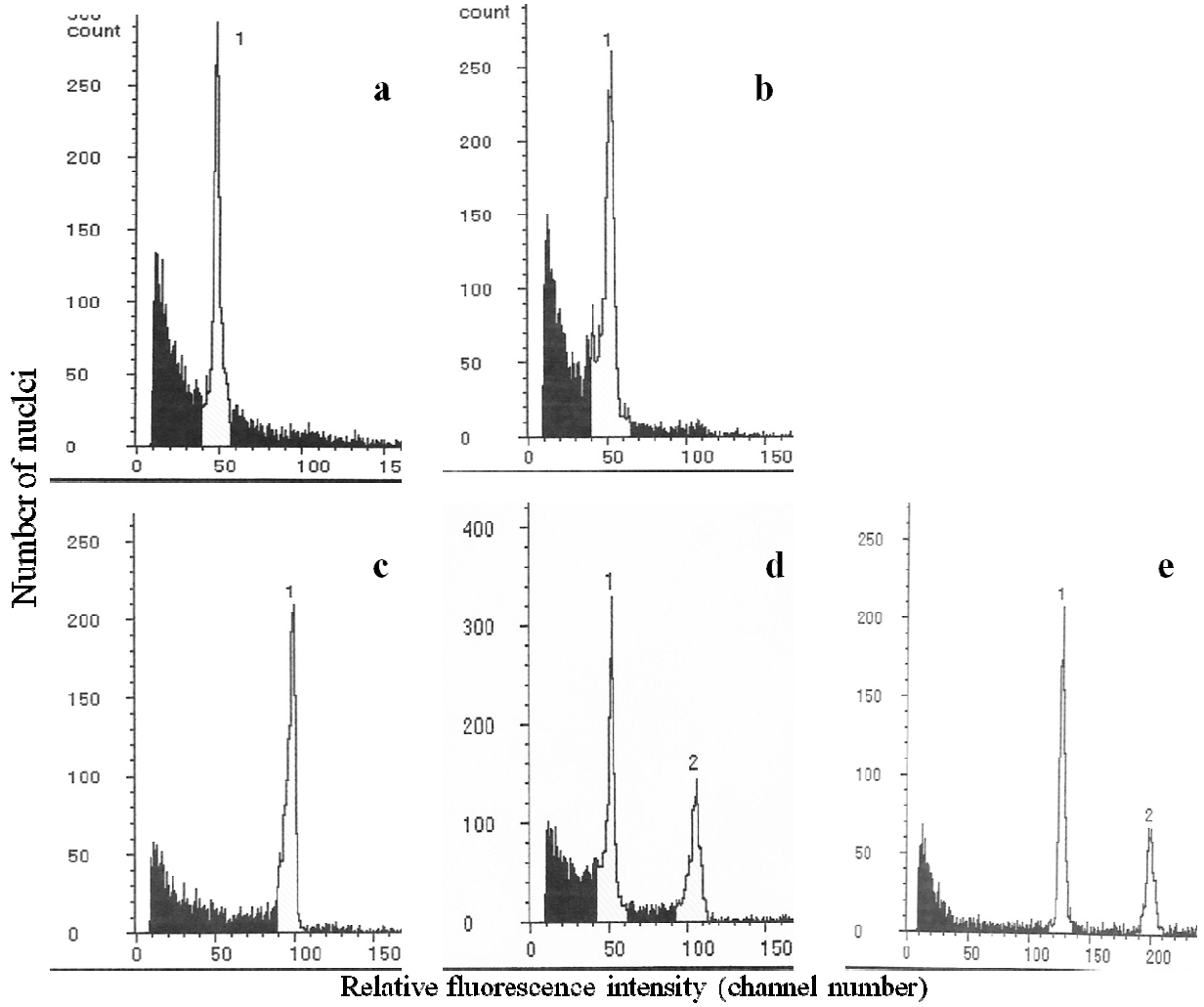

Fig.5 Flow cytometric profiles of plants regenerated from hypocotyl explants of *Parkia biglobosa* Benth. The peaks of the horizontal axis correspond to relative nuclear DNA content, which is expressed as the fluorescence intensity. The number of nuclei is shown on the vertical axis. a DNA content of leaves of control plant (diploid = 2n). b-e DNA content of leaves of regenerated plants. b diploid=2n. c tetraploid = 4n. d mixoploid = 2n + 4n. e mixoploid = 2n + 8n

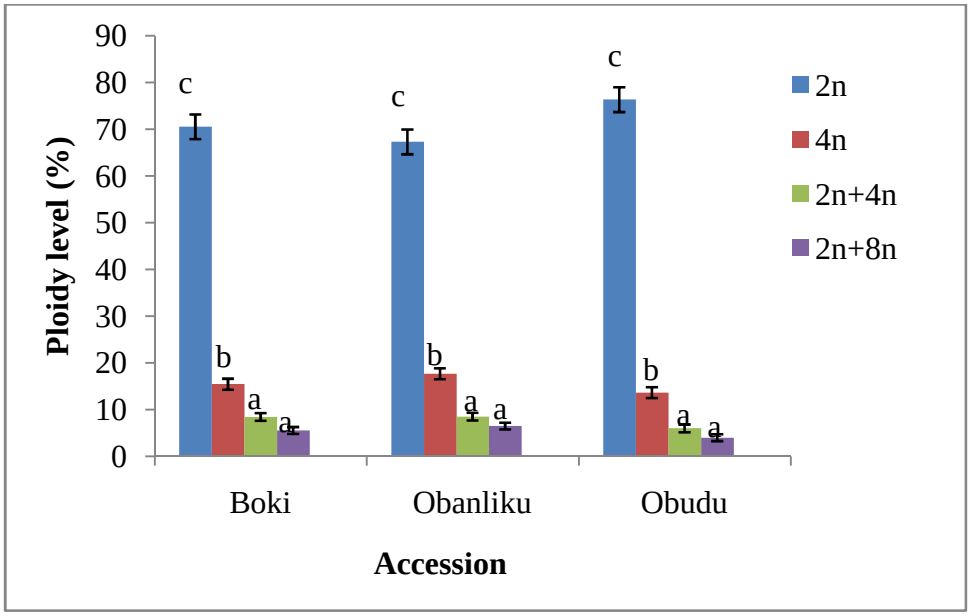

Fig. 6 Ploidy levels of plants regenerated from hypocotyl explants of *Parkia biglobosa* Benth. The ploidy of regenerants is expressed as percentage of the population tested. Values were separated using least significant difference (LSD) test at 5% level. Values with different case letters in a given accession are significantly different. 2n= diploid, 4n = tetraploid, 2n+4n = mixoploid, 2n+8n = mixoploid

Table 1.Composition of different media used for induction of callus in *Parkia biglobosa* Benth

Type	Medium composition
1	MS medium, 1 mg l^{-1} BA, 0.5 mg $l^{-1}AgNO_3$
2	MS medium, 1.5 mg l^{-1} BA, 0.5 mg $l^{-1}AgNO_3$
3	MS medium, 2 mg l^{-1} BA, 0.5 mg $l^{-1}AgNO_3$
4	MS medium, 1 mg l^{-1} BA, 0.2 mg l^{-1} NAA, 0.5 mg $l^{-1}AgNO_3$
5	MS medium, 1.5 mg l^{-1} BA, 0.2 mg l^{-1} NAA, 0.5 mg $l^{-1}AgNO_3$
6	MS medium, 2 mg l^{-1} BA, 0.2 mg l^{-1} NAA0.5 mg $l^{-1}AgNO_3$

Each medium was supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar.

CONCLUSION

The data presented here demonstrate the first successful attempt at regeneration of plantlets from hypocotyl explants of *P. biglobosa* Benth via indirect organogenesis. Further studies are needed to increase the regeneration frequency and to induce shoots from leaf explants. We hope that this protocol would provide useful information for mass propagation and germplasm conservation, genetic transformation to incorporate useful genes and for the production of valuable secondary metabolites.

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