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Full Length Research Paper

In vitro propagation of miracle berry (*Synsepalum dulcificum* Daniel) through embryo and nodal cultures

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Miracle berry is an evergreen tropical shrub which modifies sour food to produce a sweet taste. Its propagation is, however, hindered by seed recalcitrance and difficulty of stem to root. Thus *in vitro* propagation was investigated through embryo and nodal explants using different levels and combinations of auxins and cytokinins in MS medium. Embryo was regenerated in MS medium supplemented with 0.1 mg/l NAA + 0.2 mg/l BAP. Lateral buds proliferation was induced on the germinated embryo with 0.6 - 3.0 mg/l BAP + 0.1 - 0.2 mg/l NAA in which 3.0 mg/l BAP + 0.1 mg/l NAA produced highest number of buds. Rooting of the embryo regenerated plantlets was achieved with 1.0 - 2.0 mg/l IBA + 0.1 mg/l BAP. Very low (5 - 10%) axillary and terminal buds formation was achieved from nodal cultures. Few of the nodal explants formed buds with 0.1 - 0.8 mg/l NAA + 0.2 - 1.0 mg/l BAP + 0.02 mg/l GA3 with 0.8 mg/l NAA + 0.2 mg/l BAP producing the best result. However, all efforts to induce rooting on the buds formed from nodal explants proved abortive.

Key words: Miracle berry, *in vitro* conservation, recalcitrance, embryo culture, regeneration.

INTRODUCTION

Miracle berry (*Synsepalum dulcificum* Daniell) is a tropical West African shrub of the family Sapotaceae (Keay, 1992). It is reported to be indigenous to tropical West Africa and commonly found growing in the wild in fringes of virgin forest while it also grows naturally in farms and secondary bushes (Opeke, 1984). The fruits are small, approximately 2 to 3 cm long ellipsoid berries that are bright red when ripe and composed of a thin layer of edible pulp surrounding a single seed.

The most unusual thing about the fruit is the extra-ordinary effect the fleshy pulp of the fruit has on the taste buds of the tongue that causes every sour food eaten to taste very sweet. The taste-modifying effect last for 30 min to 1 h or more, causing acid food substances such as sour lime, lemon, grape fruits and even vinegar to taste sweet (Rehm and Espig, 1991). Various studies have shown that the sweetening property is due to the presence of miraculin, which is a glycoprotein, in the pulp of the berry (Metcalfe and Chalk, 1972).

The interest in natural sweeteners, which do not contain carbohydrates, has been reawakened because of the health hazards associated with the use of some artificial sweeteners like saccharine and the suspicion that these synthetic sweeteners, especially the cyclamates, are carcinogenic (WHO, 1999). The natural sweeteners of particular importance are the extremely sweet-tasting protein, monellin found in the berries of *Dioscoreophyllum cumminsii*, thaumatin from the aril of *Thaumatococcus danielli* and the miraculin from *Synsepalum dulcificum*. According to Most et al. (1979), people in parts of West Africa have been using the miracle berry to sweeten sour foods and drinks for centuries but it is only recently that the global food and pharmaceutical industries are beginning to realize its significance.

However, despite the need for large-scale production of miracle berry to exploit its potential and for further genetic studies required to enhance its improvement, commercial production of the plant has been a constraint.

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Abbreviations: MS, Murashige and Skoog; NAA, naphthalene acetic acid; BAP, 6-benzyl amino purine, IBA, indole butyric acid, 2,4-D, 2,4-dichlorophenoxylacetic acid, GA_3, gibberellic acid.

Table 1. Levels and combinations of auxin and cytokinin supplements of MS medium used in culture initiation of embryos and nodal explants of miracle berry.

Treatments	MS Medium Supplements (mg/l)
A	0.01 NAA + 0.05 BAP
B	0.1 NAA + 0.2 BAP
C	0.2 NAA + 0.5 BAP
D	0.2 NAA + 1.0 BAP
E	0.1 NAA + 0.1 BAP
F	0.2 NAA + 0.1 BAP
G	0.4 NAA + 0.1 BAP
H	0.03 NAA + 0.05 BAP
I	0.05 NAA + 0.04 BAP
J	0.8 NAA + 1.0 BAP
K	0.2 NAA
L	0.3 NAA
M	0.2 BAP
N	0.3 BAP
O	0.2 NAA + 0.3 BAP
P	20% coconut water
Q	0.05 NAA + 0.2 BAP
R	0.02 NAA + 0.05 BAP
S	10% coconut water
T	30% coconut water
U	0.5 NAA + 0.2 BAP
V	0.1 NAA + 0.3 BAP
W	0.5 2,4–D + 0.4 Kinetin
X	0.2 2,4–D + 0.1 Kinetin
Y	0.02 2,4–D + 0.01 Kinetin
Z	0 supplement (control)

Table 2. Levels and combinations of auxin and cytokinin supplements of MS medium used in shoot buds formation and proliferation of nodal cultures and regenerated embryos of miracle berry.

Treatments	MS medium supplements (mg/l)
A1	0.2 BAP
B1	0.3 BAP
C1	0.03 NAA + 0.05 BAP
D1	0.2 NAA + 0.4 BAP
E1	0.1 NAA + 0.6 BAP
F1	0.1 NAA + 1.0 BAP
G1	0.1 NAA + 3.0 BAP
H1	0.1 NAA + 4.0 BAP
I1	0.1 NAA + 5.0 BAP
J1	0.2 NAA + 4.0 BAP
K1	0.2 NAA + 5.0 BAP
L1	0.1 NAA + 6.0 BAP
M1	0.2 NAA + 3.0 BAP
N1	0.3 NAA + 4.0 BAP
Z	0 supplement (control)

The plant is recalcitrant to propagation both by seeds and cuttings, as the seeds dry very quickly after harvest and loses viability after drying (Okhapkina, 2006) while rooting of the cuttings have been very difficult. Miracle berry is also usually found growing more in the wild than cultivated and its growth rate is very slow. Hence, there is a need for an alternative method of propagation that will overcome these growth constraints. Although, some trees and shrubs have been successfully micro-propagated using Murashige and Skoog (MS) medium with minimum phytohormone modifications (Baker, 1992), there has been no report on established protocols for *in vitro* regeneration of miracle berry. The objective of this study is to regenerate plantlets of miracle berry through mature embryo and nodal cultures by determining the appropriate growth regulators (auxin-cytokinin) combinations required to modify Murashige and Skoog (MS) medium for *in vitro* propagation of the plant.

MATERIALS AND METHOD

This study was carried out in the Tissue Culture Laboratory of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Moor Plantation, Ibadan, Nigeria. Miracle berry seeds used in the embryo culture were obtained from the Institute of Agricultural Research and Training (IAR&T) Moor plantation, Ibadan. Nodal explants were from a year old seedlings obtained from Forestry Research Institute of Nigeria (FRIN) and from mature miracle berry plants at NACGRAB field gene bank, respectively.

Sterilization of materials and explants

Glasswares and dissecting tools were sterilized for 30 min by autoclaving at 121^0C and 1.06 $kgcm^{-2}$ pressure. Nodal cuttings (1.0 cm long) were surface-sterilized with 70% ethanol for 5 min, 25% solution of sodium hypochlorite (NaOCl) with 2 drops of Tween 20 for 30 min and 10% NaOCl solution for 10 min and then rinsed thrice with sterile distilled water. Miracle berry seeds, after removal of peels and fleshy pulp, were surface-sterilized with 70% ethanol for 5 min, 10% NaOCl for 20 min and 5% NaOCl for 10 min.

Media preparation

Murashige and Skoog (MS) (1962) basal medium was used in all cultures. Forty eight (48) treatments were used in all which comprise of MS basal medium without modification and MS medium supplemented with varied levels of growth regulators which include BAP, NAA, IBA, 2,4-D, kinetin and GA_3 prepared in stock solutions and used at different combinations to initiate culture, form and proliferate buds and to induce root (Tables 1, 2 and 3).

Embryo culture

Miracle berry embryos were aseptically excised from the sterilized seeds by cracking the seed coat and dissecting out the embryos with small parts of the endosperm. The embryos, which are located at the anterior end of the seeds, were immediately implanted into the culture initiation medium and 10 replicates were prepared per treatment. Also, after bud proliferation of the shoot-regenerated embryos, the regenerated shoots were excised into nodal segments and transferred into rooting medium.

Table 3. Levels and combinations of auxin and cytokinin supplements of MS medium used to induce rooting of nodal segments and shoot regenerated embryos of miracle berry.

Treatments	MS Medium supplements (mg/l)
A2	2.0 IBA
B2	3.0 NAA + 0.2 BAP
C2	0.8 NAA + 0.2 BAP
D2	2.0 NAA + 0.2 BAP
E2	2.0 IBA + 0.1 BAP
F2	1.0 IBA + 0.1 BAP
G2	3.0 IBA + 0.1 BAP
H2	5.0 IBA + 0.1 BAP
Z	0 supplement (control)

Nodal culture

Nodal explants obtained from both mature field grown miracle berry plants and one-year old screen-house raised seedlings were surface sterilized and aseptically dissected into 1.0 cm long nodal cuttings, containing at least one node with either terminal or axillary buds. These were cultured into the entire 48 MS medium modifications prepared (Tables 1, 2 and 3) with and without the addition of 0.02 mg/l of GA_3 and 20 replicates were prepared per treatment.

Growth parameter studied and data analysis

Growth parameters measured include: radicle length, shoot length, number of leaves, number of buds formed (proliferated buds); root number and root length all from the regenerated embryos while the number of terminal or auxiliary buds formed were studied from the nodal culture. Percentage embryo germination (radicle emergence), percentage rooted plantlets and the frequency of cultures that formed terminal or axillary buds were also studied. Data taken were subjected to Analysis of Variance (ANOVA) and means with significance differences separated by Duncan Multiple Range Test (DMRT) at 5% confidence level.

RESULTS

Culture initiation from mature embryo

Five out of the twenty-six different MS medium modifications supported normal germination of embryo as shown by emergence of radicles and plumules by the third week of culturing. (Table 4) MS medium supplemented with a range of 0.05 – 0.1 mg/l of NAA plus 0.04 – 0.2 mg/l BAP induced embryo culture initiation but combination of 0.1 mg/l NAA plus 0.2 mg/l BAP was shown to be optimum for morphogenesis of embryo. MS medium modified with 2,4-D and kinetin completely inhibited radicle growth and some of these hormone combinations (0.5 mg/l 2,4-D + 0.4 mg/l kinetin and 0.02 mg/l 2,4-D + 0.01 mg/l kinetin) induced callus formation that could not be induced to differentiate shoot buds. MS basal medium without modification could not support full germination of embryo because it could not induce shoot regeneration.

Lateral bud proliferation of shoot regenerated embryo

Germinated embryos transferred at six weeks to modified MS medium (Table 2) produced rapid shoot growth with bud multiplication. A range of BAP (0.6 – 3.0 mg/l) plus NAA (0.1 – 0.2 mg/l) induced node proliferation while the bud number appeared to be highest in MS medium with 3.0 mg/l BAP + 0.1 mg/l NAA in 80% of the culture (Table 5). Adventitious shoot multiplication could not be achieved through embryo culture but lateral bud proliferation was obtained through which the plant was multiplied by transferring excised nodal segments with growing bud into rooting medium.

Root formation of *in vitro* germinated embryo

Half-strength MS medium with 20 gl^{-1} sucrose without vitamin and with NAA and BAP hormone supplements (0.8 – 3.0 mg/l NAA + 0.2 mg/l BAP) induced a minor level of root formation on the germinated embryo while full-strength MS with vitamins, supplemented with 1.0 – 2.0 mg/l IBA + 0.1 mg/l BAP induced a better rooting. MS medium supplemented with 2.0 mg/l IBA + 0.1 mg/l BAP was shown to be optimum for root induction (Table 6). The plantlets from embryo cultures were acclimatized in the screen house and were successfully established on soil.

Nodal culture

From the nodal explants cultured in all the 48 MS media modifications prepared (Tables 1, 2 and 3) with and without addition of 0.02 mg/l GA_3 to initiate terminal or axillary bud formation, only three of the MS media modifications with auxin-cytokinin ratio of 0.1 – 0.8 mg/l NAA + 0.2 – 1.0 mg/l BAP and the unmodified MS medium produced minor levels of culture initiation in form of either axillary or terminal shoot bud formation (Table 7). A very low frequency of bud formation (5 – 10%) with minimum numbers of buds was observed. Also, high occurrence of microbial contamination was noticed in the nodal cultures even at 30% NaOCl sterilization and there was secretion of exudates on the nodal explants. Efforts to induce rooting on the few buds formed to regenerate whole plantlets from nodal explants were futile.

DISCUSSION

Miracle berry was successfully regenerated through mature embryo in MS medium supplemented with minimum levels of NAA, BAP and IBA. MS medium without hormonal modification could not support embryo germination of miracle berry contrary to Hartmann et al. (1997) report that *in vitro* culture of embryos does not require exoge-

Table 4. Effect of MS medium with varied hormone supplements on *in vitro* germination of mature embryos of Miracle berry.

MS Medium	Radicle Growth (%)	Morphogenesis of embryo at 6-week		
Supplement (mg/l)	(Embryo germination)	Mean radicle length (cm)	Mean shoot height (cm)	Mean leaf numbers
0.1 NAA + 0.2 BAP	100	5.0a	4.4a	4a
0.05 NAA + 0.04 BAP	80	4.0b	3.5a	3b
0.1 NAA + 0.1 BAP	60	3.2c	4.0a	3b
0.03 NAA + 0.05 BAP	30	2.5c	3.2ab	2c
0.5 NAA + 0.2 BAP	40	2.8c	2.4b	2c
MS Basal medium	20	1.8d	-	-

[1]Means with the same letter are not significantly different at 0.05 level of significance.

Table 5. Effect of modified MS medium on bud proliferation of shoot regenerated embryo of miracle berry at 6-week.

MS medium supplements (mgl^{-1})	Frequency of cultures with buds (%)	Mean no. of buds formed
0.1 NAA + 3.0 BAP	80	4a
0.1 NAA + 1.0 BAP	70	4a
0.1 NAA + 0.6 BAP	80	3b
0.2 NAA + 0.4 BAP	50	2c

[1]Means with the same letter are not significantly different at 0.05 level of significance.

Table 6. Effect of modified MS medium on root formation of *in vitro* germinated embryo of miracle berry at 5-week.

MS medium supplements (mgl^{-1})	Frequency of rooted plantlets (%)	Mean root no.	Mean root length (cm)
2.0 IBA + 0.1 BAP	90	14a	1.6b
1.0 IBA + 0.1 BAP	100	12a	1.3bc
2.0 NAA + 0.2 BAP	60	4c	0.6d
3.0 NAA + 0.2 BAP	30	3d	2.0a
0.8 NAA + 0.2 BAP	70	3d	1.1c
2.0 IBA	70	2e	0.3d
3.0 IBA + 0.1 BAP	40	2e	0.6d

[1]Means with the same letter are not significantly different at 0.05 level of significance.

Table 7. Response of nodal explants of miracle berry to bud formation and proliferation in MS medium with varied hormone supplements at 12-week.

MS medium supplements (mgl^{-1})	Frequency of culture with buds (%)	Mean no of buds formed
0.8 NAA + 0.2 BAP + 0.02 GA_3	$10^{1, 4}$	3a
0.1 NAA + 0.2 BAP + 0.02 GA_3	$5^{2, 3, 4}$	3a
0.2 NAA + 1.0 BAP	$5^{1, 4}$	2b
MS basal medium	$5^{2, 3}$	2b

[1]Terminal bud formation, [2]axillary bud formation, [3]nodal explants from mature field-grown plants, and [4]nodal explants from one-year old seedlings.
Means with the same letter are not significantly different at 0.05 level of significance.

nously supplied hormones. Embryo germination of miracle berry was achieved in MS medium modified with 0.1 mg/l NAA plus 0.2 mg/l BAP and rooting of the shoot-regenerated embryos was achieved by MS medium with 2.0 mg/l IBA plus 0.1 mg/l BAP. It was difficult to fully regenerate miracle berry through nodal explants from mature plants and young seedlings. This might be attributed to the difficulty in rejuvenating mature tissues of

woody species and production of exudates of the latex producing miracle berry.

Meanwhile, the progress made on the regeneration of whole plantlets of miracle berry from mature embryo will enhance cultivation and *in vitro* conservation of the plant that will remedy the inability to store the seeds in the gene bank due to the seed's recalcitrance to germination. It will also reduce the breeding cycle of this slow growing plant, promote germplasm movement of pathogen-free miracle berry plantlets and according to Englemann (1997), enhance germplasm diversity studies of miracle berry for the much needed characterization and classification of the plant, provide stocks for micro-grafting and promote breeding for improvement. Further studies on the growth rate under field conditions *(in vivo*) as well as further modifications of the *in vitro* physical and chemical environments of the nodal culture are recommended to enhance propagation of the plant.

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