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In vitro plant regeneration, genetic fidelity, biochemical analysis and anticancer activity of anthocyanin-rich purple flesh sweet potato var. 'Bhu Krishna'

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ABSTRACT

Purple flesh sweet potato var. 'Bhu Krishna' is a rich source of vitamins, minerals, and several important secondary metabolites, including anthocyanin. The tuber of this variety contains a high level of anthocyanin (85-90 mg/100 g). This plant is propagated through stem cutting, which is time-consuming and diseases are transmitted to the next generation. Therefore, there is an urgent need to develop a micropropagation system for the production of huge number of healthy plants to fulfil the demand of farmers as well as industrial utilization. The present study reports a reproducible micropropagation system for purple flesh sweet potato var. 'Bhu Krishna' using mature nodal explants on Murashige and Skoog's (1962) medium fortified with various plant growth regulators (PGRs). Maximum shoots regeneration was observed on MS medium fortified with 2.0 mg/l meta-Topolin. *In vitro* nodal segments were excised from established shoot cultures and inoculated on the above said optimum medium to produce more shoots (*ca.* 1300 shoots). *In vitro* regenerated shoots were successfully rooted on half-strength MS medium augmented with 0.5 mg/l Indole-3-butyric acid. The micropropagated plants were acclimatized on soil, coco peat, and sand (1:1:1) and about 95% of plants survived. Finally, these plants were transferred to the field. The genetic unifomity and phytochemical analysis of micropropagated plants vis-à-vis mother plant were demonstrated using Inter Simple Sequence Repeat markers and spectrophotometric method, respectively. The therapeutic activity in terms of anticancer activity of tubers of micropropagated plants with the mother plant was also tested. For the first time we are reporting a detailed micropropagation system for purple flesh sweet potato var. 'Bhu Krishna' for its mass propagation to fulfil the demand of farmers as well as industries.

1. Introduction

Sweet potato (*Ipomoea batatas* L.) is a perennial tuber crop belonging to the family *Convolvulaceae*. It contains a high amount of vitamins, minerals, anthocyanin, and β-carotene, (Li et al., 2019). Besides food values, it is also popular worldwide for its high nutritional values, tremondous medicinal properties, and industrial uses (Ayeleso et al., 2016). Its different plant parts, such as tuber, leaf, and stem has been used in the traditional medicines for the cure of numerous ailments. Sweet potato has been used as a remedy to cure innumerable diseases, such as anaemia, hypertension, stomach cancer, cardiovascular disease, allergies, ageing, dysentery, eye diseases, arthritis, rheumatism, etc. (Emmanuel, 2010; Pochapski et al., 2011; Mohanraj and Sivasankar, 2014; Li et al., 2019). Sweet potato leaves have been used to cure fever, nausea, splenosis, oral infections as well as Type 2 diabetes in different regions of the World, including India (Abel and Busia, 2005; Pochapski et al., 2011; Milind and Monika, 2015). In addition, leaves also possess the potential to fight against age-related macular degeneration (AMD) (Khachatryan et al., 2003; Abidin et al., 2015). Furthermore, the whole plant of sweet potato has also been reported to be used to cure dengue fever (Milind and Monika, 2015). The pharmaceutical activities of sweet potato are probably due to the presence of certain secondary metabolites,

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including anthocyanin, β-carotene, caffeoylquinic, polyphenols, etc (Ayeleso et al., 2016; Tanaka et al., 2017). Sweet potato tuber is used as raw materials in the preparation of curry, chips, bread, buns, cakes, biscuits, fish pasties, jam, ketchup, juices, and anthocyanin and β-carotene-rich curd (Owori et al., 2007; Abidin et al., 2015; Bansode et al., 2019; Bansode et al., 2020). Besides, this tuber is also rich in starch, which is useful for the preparation of starch, liquid glucose, citric acid, monosodium glutamate, and ethanol in many industries worldwide (Magoon et al., 1970; Nedunchezhiyan et al., 2012; Jena and Kar, 2019). Food and Agriculture Organisation (FAO) of the United Nations has reported that sweet potato leaves and shoots are rich in vitamins A, B, C as well as lutein. Due to being rich in nutritional content as well as wide adaptability on marginal lands, sweet potato is well known to have the potential for the prevention of malnutrition as well as increased food security in developing countries (Kang et al., 2017; Behera et al., 2022a).

Purple flesh sweet potato is a rich source of anthocyanin. Anthocyanins are an important class of flavonoids, having various health benefits which include increased longevity, cardiovascular health, cancer prevention, as well as dementia (Zhao et al., 2013; Achterfeldt et al., 2015; Liu et al., 2018; Bansode et al., 2020). About 12.5 mg of anthocyanin is required per day in daily diets of American residents (Wu et al., 2006). Thus, purple flesh sweet potato not only be used to meet people's everyday consumption of cereals and potatoes but also increase everyday consumption of anthocyanins to attain good health (Li et al., 2019).

'Bhu Krishna' is an anthocyanin rich variety of purple flesh sweet potato. The tuber of this variety contains a high amount of anthocyanin (85-90 mg/100 g) (Sunitha et al., 2018; Bansode et al., 2020). Due to its high yield (18-20 tonnes/hectore), food, medicinal, and pharmaceutical values as well as the attractive colour of the tuber, purple flesh sweet potato var. 'Bhu Krishna' is in high demand for farmers in different states of India including Odisha. This purple flesh sweet potato var. Bhu Krishna is generally propagated by the vegetative method using stem or vine cutting and storage root. Storage roots and vines are attacked by various pathogens including nematodes and insect pests (Nedunchezhiyan et al., 2012). *Meloidogyne* spp. and *Rotylenchulus reniformis* are two common nematode pests of sweet potato; they directly attach to the fibres and fleshy roots and reduce the yield and quality of the tuber (Mohandas, 2006; Behera et al., 2022a). These pathogens accumulate in vegetative propagules i.e. stem cutting and storage root and are transferred from one generation to the next generation, which leads to reduce yields and poor quality of tuber production in vegetative propagation of sweet potato (Nedunchezhiyan et al., 2012; Akomeah et al., 2019; Tadda et al., 2022). Vegetative propagation methods are not enough for constant production and supply of sufficient disease-free plants to be used as planting materials by farmers. As a result, biotechnological advances through plant tissue culture has served itself as a reliable and promising tool to overcome these problems by producing a huge number of healthy, clonally, and biochemically stable plants (Behera et al., 2022a). But the chance of somatic variations of micropropagated plants cannot be avoided, especially with the use of different types plant growth regulators (PGRs), long time maintainance of culture and repeated subculture, etc., which may induce changes in the plants at its phenotypic or genetic or biochemical level (Behera et al., 2019). Thus, the evaluation of genetic fidelity and biochemical analysis of micropropagated plants are also very important. Herein, we develop an efficient and reproducible micropropagation system using nodal segment for the production of large number of genetically and biochemically stable plants of anthocyanin-rich purple flesh sweet potato var. 'Bhu Krishna'. The anticancer activity was also tested in the tuber extracts of both mother plant and micropropagated plants against two cell lines i.e. MCF-7 and MDA-MB-231.

2. Materials and methods

2.1. Preparation of plant materials

Healthy purple flesh sweet potato var. 'Bhu Krishna' plants is a clonal selection from germplasm line ST-13 (IC 597029) maintained at the gene bank of ICAR-Central Tuber Crops Research Institute, Bhubaneswar, Odisha, India (Fig. 1a). Young and healthy stems were collected from the experimental field. Stems were washed properly in tap water, the leaves were removed and stems were cut into small pieces to be used as explants. Each explant contains one nodal region (1.0-1.5 cm). Then the explants were pre-sterilised with 1.0% (v/v) Tween-20 for 15 min followed by 2.0% (w/v) 'Bavistin' treatment for 15 min and then 3-5 times washed with double distilled water. Then the nodal explants were sterilised with 0.1% (w/v) mercuric chloride for 5 min followed by 3-5 times washed with sterile distilled water. Subsequently, explants were treated with 70% ethanol for 2 min and rinsed 3 times with sterile double distilled water. Finally, the nodal segments were trimmed on both sides and used for micropropagation experiments (Fig. 1b).

2.2. Multiple shoots proliferation

Nodal segments were inoculated on PGRs free Murashige and Skoog's (1962) (MS) medium and MS medium with various PGRs i.e. N^6-benzyladenine (BA), Kinetin (KIN), Zeatin (Z), and meta-Topolin (mT) each with 0.5 mg/l to 5.0 mg/l were used. Further, MS medium having 2.0 mg/l mT fortified with different auxins including α-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), and Indole-3-acetic acid (IAA) each with 0.5 mg/l to 1.5 mg/l were also tested for shoot proliferation. In all culture medium, 3% sucrose was used and CleriGel (0.2%; w/v) was used as gelling agent. The medium pH was attuned to 5.8 ± 0.1 before autoclave.

In vitro shoots proliferated from the nodal segments were excised and cut into small pieces, each containing one nodal region. They were then cultured on the optimum shoot proliferation medium (MS + 2.0 mg/l mT, as evaluated earlier from shoot proliferation experiment using mature nodal segments) for large scale shoot proliferation in a short duration. The mother nodal segments, after harvesting of shoots, were repeatedly sub-cultured on the same culture medium i.e. MS + 2.0 mg/l mT for further shoot multiplication. All the cultures were maintained at 25±1°C with 16 h/ 8 h photoperiod and 50 μmol m^{-2} s^{-1} light intensity.

2.3. Rooting of shoots

Shoots regenerated *in vitro* (3.0-4.0 cm) from nodal segments were excised and inoculated on half-strength MS ($^1/_2$ MS) medium and $^1/_2$ MS medium with 0.3 to 2.0 mg/l IBA for rooting.

2.4. Acclimatization of plantlets

The well rooted shoots were carefully removed from the culture tubes. The roots were washed thoroughly with tap water to remove the adhered agar media. The plants were planted in the multiple potting trays containing soil, coco peat, and sand (1:1:1) and kept inside the green house. The potting mixtures were soaked with normal tap water. The plantlets were watered as per the requirement by checking every day. After two weeks of planting, plants were shifted to a shade house from the greenhouse. In the shade house the plants were kept for another 2 weeks and watered at 2 days interval. Then the plants were shifted to pots containing the soil, sand, and cow dung (1:1:1) and kept in a shade house for 1 week, then transferred to sunlight. Finally, the acclimatized plants were transferred to the field.

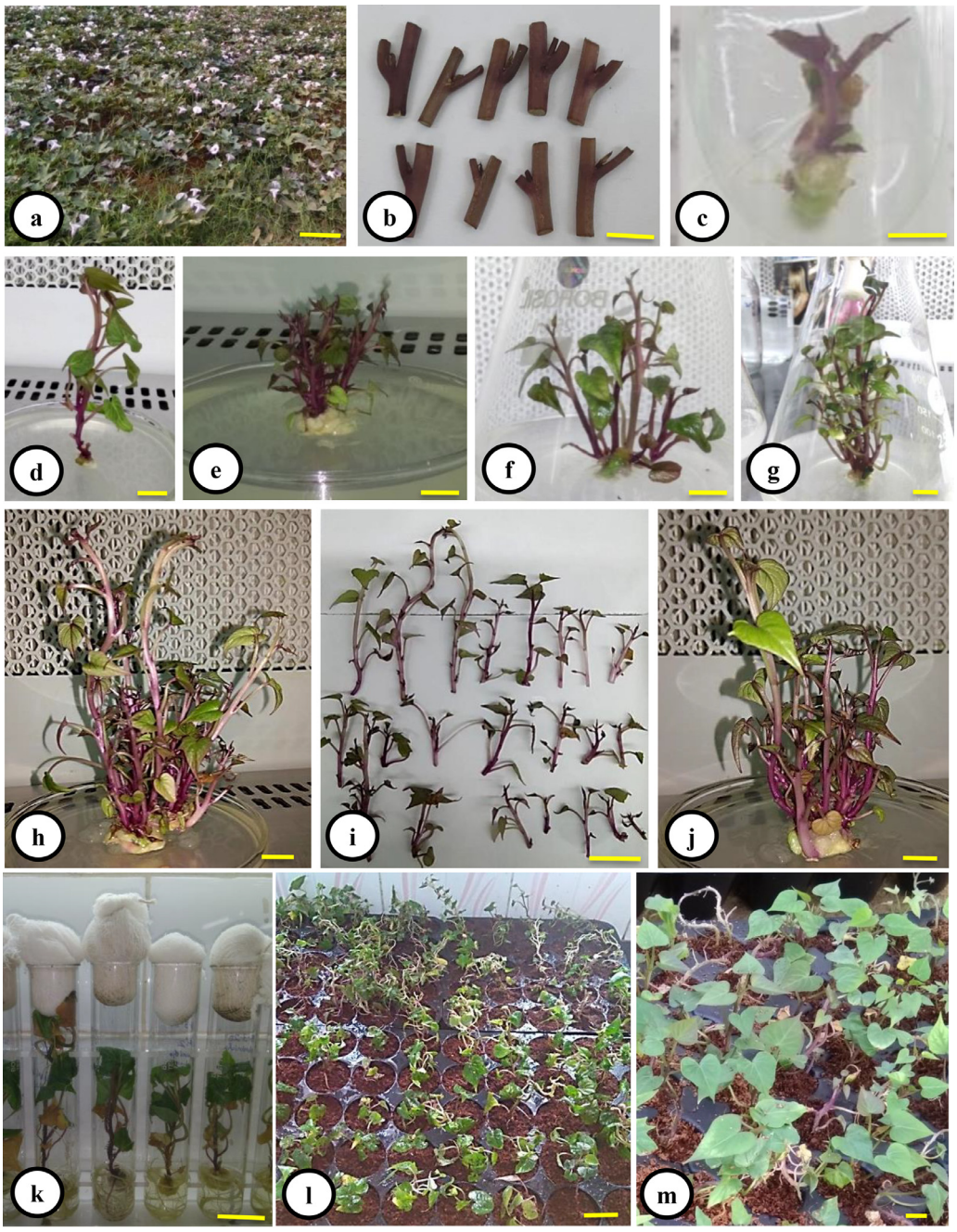

Fig. 1. Micropropagation of purple flesh Sweet potato var. Bhu Krishna: (a) Plant growing in the field, (b) Nodal segments of purple flesh sweet potato var. Bhu Krishna, (c) Shoot bud initiation from the nodal segment, (d) Single shoot regenerated from nodal segment on MS medium, (e) Multiple shoot formation on MS medium supplemented with 2.0 mg/l BA after six weeks of culture, (f) Multiple shoot formation and elongation from the nodal segment of sweet potato on MS + 3.0 mg/l KIN, (g) Multiple shoot formation and elongation from the nodal segment on MS + 3.0 mg/l Z, (h) Multiple shoot formation and elongation from the nodal segment on MS + 2.0 mg/l mT, (i) *In vitro* shoot were harvested from mother explant, (j) Multiple shoot formation and elongation from *in vitro* nodal segment on MS + 2.0 mg/l mT, (k) Rooting of *in vitro* regenerated shoot on $^{1}/_{2}$ MS + 0.5 mg/l IBA, (l & m) Acclimatization of micropropagated plants in multiple potting tray. Bars: 1 cm.

2.5. Genetic uniformity assessment

For the genetic unifomity study, randomly thirteen *in vitro* raised plants (plants regenerated on MS medium supplemented with 2.0 mg/l mT) were selected. Fresh young leaves were collected from the mother plant and micropropagated plants. The total genomic DNA was isolated and purified using the protocol described by Doyle and Doyle (1990). Both quality and quantity of genomic DNA were verified on 0.8% agarose gel by comparing with uncut λ DNA band intensities. For preliminary screening, twenty ISSR primers were used initially and then twelve primers were selected out of twenty primers for final amplification based on the amplification of clear bands. The primer amplification was performed using the standard protocol reported by Behera et al. (2018).

2.6. Biochemical analysis of micropropagated plant tubers

2.6.1. Sample preparation

Tubers were collected from the mother plant and micropropagated plants (plants regenerated on MS medium supplemented with 2.0 mg/l mT) and cleaned properly. Tubers were sliced and dried under shade conditions at room temperature to get constant weight.

Then, these tuber samples were powdered and kept in an air tight ampule in a dark place for use in biochemical analysis.

2.6.2. Estimation of flavonoids, saponins, phenolics, tannins, alkaloids, and anthocyanins contents

The tuber samples of both the micropropagated plants and the mother plant were tested for phytochemical estimation. The phytochemicals i.e., alkaloids, flavonoids, phenolics, tannins, and saponins contents of tuber samples were estimated following the procedure reported by Sutar et al. (2020). Whereas, anthocyanin quantity was estimated following protocol reported by Ghasemzadeh et al. (2016).

2.6.3. Estimation of starch, and sugar

Starch content of tuber was estimated using anthrone reagent method followed by protocol as described by McCready et al. (1950). Sucrose content was estimated following the phenol-sulfuric acid method as reported by Dubois et al. (1956).

2.7. Anticancer activity study

Anticancer activity in terms of cell antiproliferative activity assay and apoptosis assay of micropropagated plants (plants regenerated on MS medium supplemented with 2.0 mg/l mT) and mother plant tuber extracts (methanol and aqueous extracts) was executed following standard procedure reported earlier by Meher et al. (2021) and Behera et al. (2022b; 2023a) against two cell lines i.e. MCF-7 and MDA-MB-231. Apoptotic cancer cells were observed in fluorescence microscope separately using different staining and identified by the change of morphological features.

2.8. Statistical analysis of data

Entire experiments were conducted thrice. Thirty replicas (culture vessels) were taken in tissue culture experiments and one explant per culture vessel. The data was documented at one-week intervals. The final data were expressed as the mean number of shoots/nodal segment, roots/shoot, shoot length, and root length after six weeks. Statistical data analysis was done using Duncan's multiple new range tests (DMRT) following the statistical method described by Gomez and Gomez (1984). For phytochemicals data analysis T-test was used.

3. Results

3.1. Multiple shoots proliferation

Nodal segments were inoculated on PGRs free MS medium and MS medium with various PGRs. Shoot initiation was shown after one week of inoculation on all the tested media (Fig. 1c). The percentage of explant responded and number of shoot regenerated per explant varied from medium to medium (Table 1). About 53.3% of explants responded on PGRs free MS medium. On MS medium only 1.0 shoot per explant was produced having shoot length of ca. 5.3 cm (Fig. 1d). Among the different cytokinins tested, on BA 2.0 mg/l supplemented MS medium 83.3% of explant responded for shoot regeneration. On this medium about 5.5 shoots with mean shoot length 4.2 cm was found (Fig.1 e). On 3.0 mg/l KIN about 77.8% of explant responded and 4.8 shoots with an average shoot length of 4.5 cm was found (Table 1; Fig. 1f). Similarly, about 88.9% of explants responded on MS medium with Z (3.0 mg/l). On MS+3.0 mg/l Z medium, 7.5 shoots were recorded with mean shoot length 5.0 cm (Fig. 1g). Maximum percentage of explant response (95.6%) was observed on MS medium with mT (2.0 mg/l). On this medium highest numbers of shoot proliferation (20.3) with average shoot length 5.3 cm was also reported within six weeks of culture (Table 1; Fig. 1 h).

For up-scaling of shoots, primary shoots were excised from the starting (mother) explant (Fig. 1i) and cut into small pieces and (an average of 5 nodal segments were found from one shoot) about 100 nodal segments were obtained from 20.3 shoots. These *in vitro* nodal explants were cultured on MS + 2.0 mg/l mT medium, which was recorded as the optimum shoot proliferation medium for mature nodal segments. All explants were responded to this medium and about 13.0 number of shoots with 5.0 cm shoot length were produced within six weeks of culture (Fig. 1j). In this way, *ca.* 1300 shoots were obtained from a single nodal segment within twelve weeks. To evaluate the shoot regeneration potential of the mother explants (mature nodal segments), after harvesting the *in vitro* primary shoots, the mother nodal explants were cultured on MS medium + 2 mg/l mT medium. The multiple shoot formation ability of the mother explants increased untill 3rd sub-culture, during which about 24.5 number of shoots were produced. After the 3rd sub-culture, the number of multiple shoots regeneration potential from the mother explants significantly reduced (Fig. 2).

3.2. In vitro *rooting of shoots*

In vitro shoots (3.0-4.0 cm) were excised and cultured on $^{1}/_{2}$ MS medium and $^{1}/_{2}$ MS medium with 0.3-2.0 mg/l of IBA. The rooting percentage of *in vitro* regenerated shoots varied with the rooting medium used (Table 2). The addition of auxin to the basal MS medium was essential for root initiation from the *in vitro* shoots. Highest per cent of rooting (97.8%) of shoots were recorded on $^{1}/_{2}$ MS with 0.5 mg/l IBA. On this medium, about 5.8 roots/shoot having mean root length of 5.5 cm was recorded (Fig. 1k; Table 2).

3.3. Acclimatization of plantlets

Approximately 95% of *in vitro* regenerated plants survived the acclimatzation process of this study where coco peat, sand, and soil (1:1:1) were used in the potting tray (Fig. 1l & m). Subsequently, the plants were successfully transferred to larger pots containing soil, cow dung, and sand (1:1:1) and all the acclimatized plants shifted to the field conditions survived.

3.4. Genetic uniformity analysis

Genetic uniformity analysis of micropropagated plants regenerated on MS medium supplemented with 2.0 mgl mT in comparison with the mother plant was done using ISSR primers. In this study, twenty primers were screened during the preliminary study and finally, twelve primers were selected based on clear amplified bands productions by primers. Total 55 bands were amplified by twelve ISSR markers. An average number of bands was 4.58. Amplified bands ranges from 300 to 2400 bp (Table 3). Maximum number of bands (8) were observed in primer Oligo 11(b) followed by 6 number of bands were observed in ISSR 1, ISSR 27, and Oligo 4(b) (Table 3; Fig. 3a-d). All amplified bands were monomorphic patterns indicating the genetic stability of the micropropagated plants (Fig. 3 a-d).

3.5. Biochemical analysis

Biochemical analysis in terms of alkaloids, anthocyanin, flavonoids, phenolics, saponins, tannins, sugar, and starch contents was estimated in the tuber sample of both micropropagated plants vis-à-vis mother plant. In this study no significant difference was observed in the quantity of phytochemicals present in the tuber of the micropropagated plants and mother plant (Table 4). Among all the tested phytochemicals, as expected, starch was found to be in maximum amounts. There was no significant difference among the starch content of the tuber of the mother plant (162.29 mg/g DW) and micropropagated plants (161.96 mg/g DW). Anthocyanin, which is an important phytochemical in this variety of sweet potato, was found to be almost similar quantity in the tuber of mother plant (0.872 mg/g DW) and micropropagated plant (0.870 mg/g DW) (Table 4).

Table 1
Effect of growth regulators on *in vitro* multiple shoot proliferation from nodal segments of purple flesh sweet potato var. Bhu Krishna

BA	KIN		mT	NAA	IBA	IAA	Response of explants (%)	Mean shoots/explant	Shoot length (cm)
-	-		-	-	-	-	53.3 ± 0.5^{s}	1.0 ± 0.0^{z}	5.3 ± 0.3^{a}
0.5	-	-	-	-	**MS medium with plant growth regulators (mg/l)**	-	65.6 ± 1.0^{o}	2.5 ± 0.5^{xy}	3.5 ± 0.2^{gh}
1.0	-	-	-	-	**Z**	-	76.7 ± 1.2^{ij}	$3.8 \pm 0.3^{t-v}$	$4.0 \pm 0.1^{d-f}$
2.0	-	-	-	-	-	-	83.3 ± 0.9^{g}	5.5 ± 0.5^{pq}	4.2 ± 0.2^{de}
3.0	-	-	-	-	-	-	80.0 ± 0.5^{h}	5.0 ± 0.2^{qr}	4.2 ± 0.3^{de}
4.0	-	-	-	-	-	-	73.3 ± 0.5^{lm}	$4.4 \pm 0.4^{r-t}$	$3.8 \pm 0.2^{e-g}$
5.0	-	-	-	-	-	-	67.8 ± 0.3^{n}	$3.0 \pm 0.0^{v-x}$	2.5 ± 0.1^{j}
-	0.5		-	-	-	-	58.9 ± 0.8^{r}	1.0 ± 0.0^{z}	3.5 ± 0.2^{gh}
-	1.0		-	-	-	-	63.3 ± 0.6^{pq}	2.5 ± 0.3^{xy}	$3.8 \pm 0.4^{e-g}$
-	2.0		-	-	-	-	74.4 ± 0.3^{kl}	$4.0 \pm 0.4^{s-u}$	4.2 ± 0.3^{de}
-	3.0		-	-	-	-	77.8 ± 1.4^{i}	$4.8 \pm 0.4^{q-s}$	$4.5 \pm 0.4^{b-d}$
-	4.0		-	-	-	-	73.3 ± 0.7^{lm}	$4.0 \pm 0.0^{s-u}$	$4.0 \pm 0.5^{d-f}$
-	5.0		-	-	-	-	73.3 ± 0.4^{lm}	$3.5 \pm 0.1^{u-w}$	3.5 ± 0.1^{gh}
-	-	0.5	-	-	-	-	64.4 ± 0.7^{op}	$3.5 \pm 0.2^{u-w}$	$3.8 \pm 0.2^{e-g}$
-	-	1.0	-	-	-	-	75.6 ± 0.9^{jk}	$4.0 \pm 0.2^{s-u}$	4.2 ± 0.4^{de}
-	-	2.0	-	-	-	-	83.3 ± 1.1^{g}	6.3 ± 0.5^{p}	$4.8 \pm 0.2^{a-c}$
-	-	3.0	-	-	-	-	88.9 ± 0.9^{d}	7.5 ± 0.3^{n}	5.3 ± 0.3^{a}
-	-	4.0	-	-	-	-	85.6 ± 0.5^{f}	7.4 ± 0.4^{no}	5.0 ± 0.1^{ab}
-	-	5.0	-	-	-	-	77.8 ± 1.1^{i}	$4.8 \pm 0.3^{q-s}$	$4.0 \pm 0.0^{d-f}$
-	-	-	0.5	-	-	-	75.6 ± 0.3^{ij}	11.3 ± 0.5^{kl}	3.5 ± 0.2^{gh}
-	-	-	1.0	-	-	-	87.8 ± 0.5^{de}	15.6 ± 0.5^{fg}	$4.8 \pm 0.1^{a-c}$
-	-	-	2.0	-	-	-	95.6 ± 1.2^{a}	20.3 ± 0.6^{a}	5.3 ± 0.0^{a}
-	-	-	3.0	-	-	-	93.3 ± 2.2^{b}	16.5 ± 0.5^{ef}	5.0 ± 0.3^{ab}
-	-	-	4.0	-	-	-	93.3 ± 1.2^{b}	13.8 ± 0.9^{hi}	$4.5 \pm 0.1^{b-d}$
-	-	-	5.0	-	-	-	85.6 ± 1.0^{f}	10.0 ± 0.4^{m}	$3.8 \pm 0.0^{e-g}$
-	-	-	2.0	0.5	-	-	87.8 ± 0.8^{de}	14.5 ± 0.9^{h}	$4.0 \pm 0.4^{d-f}$
-	-	-	2.0	1.0	-	-	87.8 ± 1.4^{de}	12.0 ± 0.7^{jk}	$3.8 \pm 0.2^{e-g}$
-	-	-	2.0	1.5	-	-	80.0 ± 1.0^{h}	12.8 ± 0.5^{j}	3.0 ± 0.1^{i}
-	-	-	2.0	-	0.5	-	95.6 ± 1.2^{a}	18.0 ± 1.0^{bc}	4.2 ± 0.3^{de}
-	-	-	2.0	-	1.0	-	95.6 ± 0.5^{a}	18.7 ± 0.7^{b}	$4.5 \pm 0.3^{b-d}$
-	-	-	2.0	-	1.5	-	91.1 ± 0.9^{c}	16.5 ± 0.9^{ef}	$4.0 \pm 0.1^{d-f}$
-	-	-	2.0	-	-	0.5	87.8 ± 0.3^{de}	$17.9 \pm 0.7^{b-d}$	$4.8 \pm 0.3^{a-c}$
-	-	-	2.0	-	-	1.0	87.8 ± 0.2^{de}	17.0 ± 0.6^{de}	4.2 ± 0.2^{de}
-	-	-	2.0	-	-	1.5	83.3 ± 0.5^{g}	15.6 ± 0.5^{fg}	$4.0 \pm 0.1^{d-f}$

All values in the column represented as means ± standard deviation (SD). In a column, same letters written in superscripts indicate not significant difference among the means (P≤0.05; DMRT)

3.6. Anticancer property study

Anticancer property in terms of cell antiproliferative activity of methanol and aqueous extract of the tuber of the mother plant and micropropagated plant was carried out against cell lines such as MCF-7 and MDA-MB-231. The IC_{50} concentration of aqueous extract of the mother plant tuber and micropropagated plant tuber were found to be 56.0 μg/ml and 72.6 μg/ml respectively, against MCF-7 cell lines. For MDA-MB-231 cell line, the IC_{50} concentration of aqueous extract of mother plant tuber and micropropagated plant tuber were found at 86.7 μg/ml and 99.7 μg/ml respectively (Table 5). The methanol extract was also tested against both the cell line i.e., MCF-7 and MDA-MB-231 and found that the mother plant tuber IC_{50} concentration 68.1 μg/ml and micropropagated plant tuber 76.5 μg/ml against MCF-7 cell line. Whereas, the IC_{50} concentration of methanol extract of the mother plant and micropropagated plant tuber was found 92.2 μg/ml and 93.7 μg/ml respectively against MDA-MB-231 cell line (Table 5).

The study on apoptosis test was performed using IC_{50} concentrations of both methanol and aqueous tuber extracts of the mother plant and micropropagated plant against the MDA-MB-231 cell line. The cell death due to apoptosis was morphologically identified using

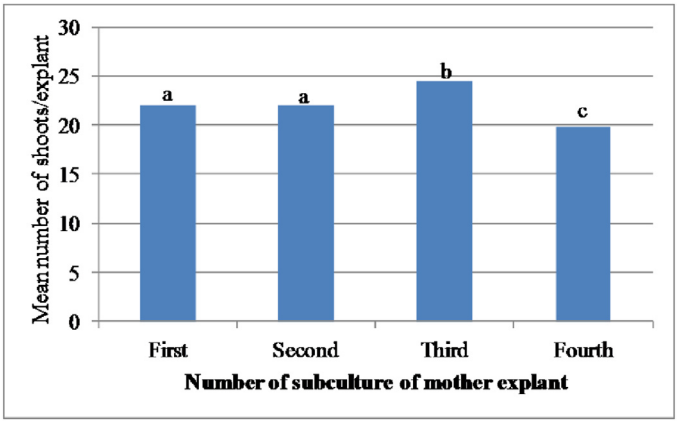

Fig. 2. *In vitro* shoot regeneration from mother explant after repeated subculture on MS + 2.0 mg/l mT. In graph, columns with same letters indicate statistically not significant among the means (p≤0.5; Duncan's multiple range test).

Table 2
In vitro rooting of shoots derived from nodal segments of purple flesh sweet potato var. Bhu Krishna

$^1/_2$ MS medium with IBA (mg/l)	Response of shoot for rooting (%)	Mean no. of roots/ shoot	Mean root length (cm)
0.0	65.6±1.0^{e}	3.0±0.3^{e}	5.3±0.3^{b}
0.3	88.9±1.8^{bc}	4.3±0.2^{c}	4.7±0.2^{cd}
0.5	97.8±1.7^{a}	5.8±0.2^{a}	5.5±0.3^{a}
1.0	90.0±2.0^{b}	5.0±0.3^{b}	5.3±0.4^{b}
1.5	83.3±0.5^{d}	3.5±0.1^{d}	5.0±0.3^{bc}
2.0	65±1.5^{ef*}	1.0±0.0^{f}	3.0±0.1^{e}

All values in the column represented as means ± standard deviation (SD). In a column, same letters written in superscripts indicate not significant difference among the means (P≤0.05; DMRT).
* Basal callus at the end of shoot

Table 3
Details of ISSR primers used for genetic fidelity of micropropagated plants of purple flesh sweet potato var. 'Bhu Krishna'.

Name of Primers	Primer sequences (5'-3')	Mt (°C)	At (°C)	No. of scorable bands	Approximate range of amplified bands (bp)
ISSR 1	$(GA)_8G$	57.2	52.2	6	1000-2100
ISSR 2	$(AG)_8G$	57.2	52.2	3	500-1250
ISSR 13	$C(GATA)_4$	47.5	42.5	4	550-1400
ISSR 27	$(GT)_6CA$	49.7	44.7	6	800-2100
ISSR 32	$(CTG)_9G$	74.9	69.9	2	700-1600
ISSR 33	$(GCA)_8AG$	72.5	67.5	5	300-1500
ISSR 37	$(ACA)_5CT$	50.0	45.0	4	400-1600
ISSR 40	$(GAA)_6$	50.8	45.8	3	400-1400
Oligo4(a)	$(GACA)_4T$	50.0	45.0	4	500-1300
Oligo4(b)	$T(GACA)_4$	50.0	45.0	6	900-2100
Oligo11(a)	$G(CTGT)_4$	52.0	47.0	4	500-1200
Oligo11(b)	$(CTGT)_4C$	52.0	47.0	8	350-2400

Mt- melting temperature; At- annealing temperature

DAPI staining, Acridine orange (AO) staining, and Ethidium bromide (EtBr) staining. All these stainings showed apoptotic cell death of the MDA-MB-231 cell line, which is characterized by chromatin condensation, plasma membrane blebbing, and appearance of apoptotic bodies with the treatment of methanol and aqueous extracts (Fig. 4).

4. Discussions

4.1. Plant regeneration

Plant regeneration via plant tissue culture system has several advantages over the conventional method of propagation through seed, cuttings, grafting as well as air layering (Suman, 2017). Tissue culture is a rapid propagation process which has the ability to regenerate huge number of healthy plants without depending on any seasonal and climatic condition (Garcia-Gonzales et al., 2010). Utilization of the best agronomic practices does not give the highest yield without using healthy and disease-free planting material (Chatenet et al., 2001; Behera et al., 2022a). Nowadays, the horticultural and herbal industry is revolutionized by adopting tissue culture techniques as a viable alternative propagation method that have potential to regenerate huge number of disease-free stock materials and a consistent supply of good quality planting materials (Behera et al., 2022a). Many *in vitro* regenerated important horticultural plants, including *Psidium guajava* (Rai et al., 2010; Singh and Singh, 2018; Gollagi et al., 2019), *Punica granatum* (Naik and Chand, 2011; Patil et

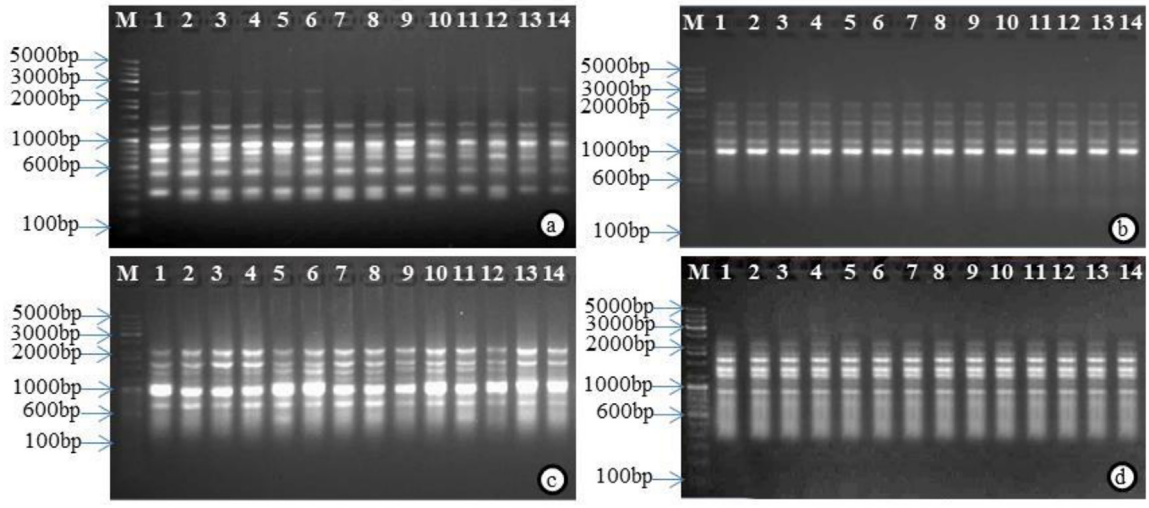

Fig. 3. DNA banding pattern of mother plant and micropropagated plants of purple flesh Sweet potato var. Bhu Krishna using ISSR primers: (a) Oligo 11(b), (b) ISSR1, (c) ISSR 27, and (d) Oligo 4(b). M is the marker; Lane 1 mother plant, Lane 2-14 *in vitro* regenerated plants.

Table 4
Phytochemical composition of tuber sample of mother vis-à-vis *in vitro* regenerated plant of purple flesh sweet potato var. Bhu Krishna and other varieties/cultivars of sweet potato.

Sweet potato varieties/ cultivars	Phytochemicals							
	Alkaloids (mg/g DW)	Anthocyanins (mg Cy3GE/g DW)	Flavonoids (mg/g DW)	Phenolics (mg GAE/g DW)	Saponins (mg/g DW)	Sugar (mg/g DW)	Starch (mg/g DW)	Tannins (mg TAE/g DW)
*Mother plant tuber of sweet potato var Bhu Krishna	0.431± 0.002 [a]	0.872 ±0.002 [a]	0.583± 0.003 [a]	0.748 ± 0.007 [a]	2.120 ± 0.010 [a]	18.230 ±0.020 [a]	162.291 ±2.645 [a]	12.420 ± 2.00 [a]
**In vitro* regenerated plant tuber of sweet potato var Bhu Krishna	0.427± 0.004 [a]	0.870 ±0.005 [a]	0.579 ± 0.000 [a]	0.743 ± 0.003[a]	2.100 ± 0.01 [a]	18.150 ±0.052 [a]	161.962 ±1.732 [a]	12.340 ± 0.069 [a]
(Shekhar et al., 2015)[1]Tuber of orange flesh sweet potato cv SP-6	0.016	-	0.705	1.49	-	27.6	558.3	-
[1]Tuber of white flesh sweet potato	0.012	-	0.617	1.58	-	44.7	432.5	-
[2]Tuber of purple colour sweet potato cv G-0615	-	-	-	-	66.0	8.9	-	-
[3]Tuber of sweet potato var K/KA/ 2004/215	-	-	0.2585	1.905	-	-	-	-
[3]Tuber of sweet potato var Kens-pot 4	-	-	0.0139	1.0368	-	-	-	-
[3]Tuber of sweet t potato var Kenspot 5	-	-	0.0103	1.3979	-	-	-	-
[3]Tuber of sweet potato var Kabode	-	-	0.2181	0.9518	-	-	-	-
[3]Tuber of sweet potato var SPK031	-	-	0.2581	2.2355	-	-	-	-
[3]Tuber of sweet potato var SPK4	-	-	0.0421	0.9275	-	-	-	-
[3]Tuber of sweet potato var Vitaa	-	-	0.1263	1.7672	-	-	-	-
[3]Tuber of sweet potato var Yellowsp	-	-	0.1263	1.8742	-	-	-	-
[4]Tuber of sweet potato	18.8	17.1	63.3	44.0	20.1	-	-	2.2
[5]Tuber of sweet potato var Jizi 01	-	6.23	-	54.3	-	-	686.0	-
[5]Tuber of sweet potato var Xinong 431	-	2.56	-	25.7	-	-	601	-
[5]Tuber of sweet potato var Bei-jing 553	-	1.32	-	17.8	-	-	702	-
[5]Tuber of sweet potato var Shangshu 19	-	UD	-	9.6	-	-	714	-

All values in the column represented as means ± standard deviation (SD). In a row, same letters written in superscripts indicated not statistically significant difference between the means ($P \leq 0.05$; t- test).
DW: dry weight, Cy3-GE: cyanidin 3-glucoside equivalent, GAE: gallic acid equivalent, TAE: tannic acid equivalent, UD: Undetectable value.

* Present study
[1] Shekhar et al. (2015)
[2] Drapal et al. (2019)
[3] Abong et al. (2020)
[4] Agubosi et al. (2021)
[5] Ji et al. (2015).

Table 5
IC_{50} values of aqueous and methanol extract of of purple flesh sweet potato var. 'Bhu Krishna' tuber extract against two cell lines i.e., MCF-7 and MDAMB-231.

Tuber extracts	IC_{50} (μg/ml)			
	MCF-7		MDA-MB-231	
	Mother plant	Tissue culture plant	Mother plant	Tissue culture plant
Aqueous extract	56.0±3.6	72.6±4.5	86.7±4.4	99.7±3.6
Methanol extract	68.1±3.5	76.5±3.4	92.2±4.3	93.7±2.5

Values represent means ± standard deviation (SD).

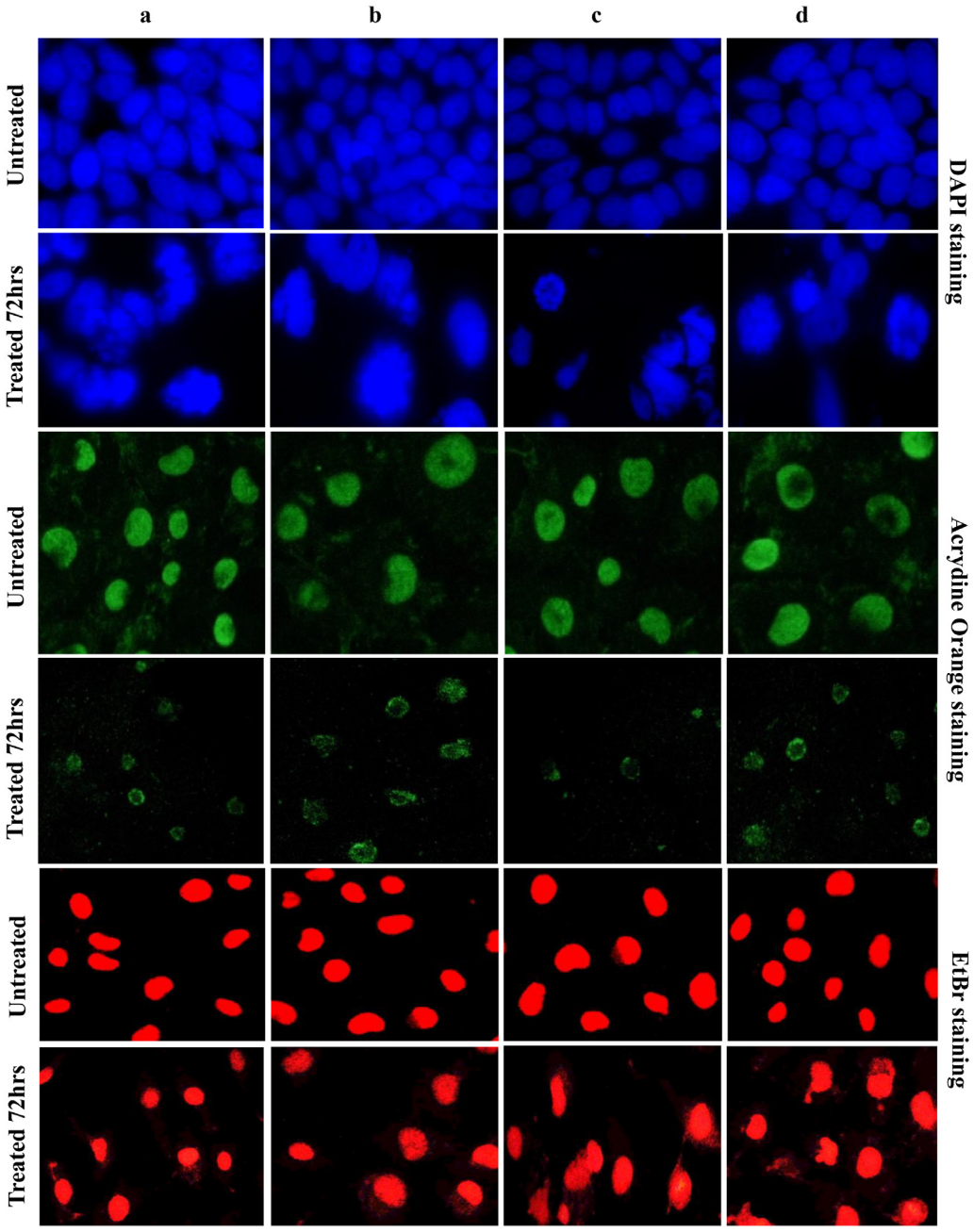

Fig. 4. Morphological characters like chromatin condensation, blebbing of membrane, apoptotic bodies of MDA-MB-231cells shown using fluorescence microscopy stained with DAPI, Acridine Orange, and EtBr. Cells treated with (IC_{50}) value of the mother plant and micropropagated plant tuber extract; (a) mother plant methanol extract, (b) micropropagated plant methanol extract, (c) mother plant aqueous extract, and (d) micropropagated plant aqueous extract for 72 hours.

al., 2011), *Fragria ananassa* (Kadhimi et al., 2014; Naing et al., 2019), *Dioscorea alata* (Alizadeh et al., 1998; Jasik and Mantell, 2000; Borges et al., 2005; Fotso et al., 2013), *Hedychium coronarium* (Behera et al., 2023a), *Ipomoea batatas* (Frison and Ng, 1981; Oggema et al., 2007) were established by using tissue culture technique. In this study, we developed an efficient *in vitro* plant regeneration system for anthocyanin-rich purple flesh sweet potato var. 'Bhu Krishna' for first time using nodal explant. 'Bhu Krishna' is an anthocyanin rich (85-90 mg/100g) sweet potato variety. This variety is cultivated by using stem cuttings or tubers, which is slow, time cosuming, as well as labourious process. Besides, the stem cutting or tuber are attacked by disease causing pathogens (*Meloidogyne* spp. and *Rotylenchulus reniformis*) leads to decreases the yield of tuber quantity and quality also (Nedunchezhiyan et al., 2012; Tadda et al., 2022).

For the development of a simple and efficient micropropagation protocol, there is a need to evaluate a number of factors including medium and PGRs, etc. In this study mature nodal segment was chosen as explant and preferred for *in vitro* plant regeneration of 'Bhu Krishna' as nodal segments are known to have least possibility to somaclonal variation compare to unorganized callus (Mohanty et al., 2013). The nodal segments/ explants have already been used as explants for micropropagation of not only *Ipomoea batatas* (Liz and Conover, 1978; Dodds et al., 1991; Mukherjee, 2002; Yang, 2010; Dolinski and Olek, 2013; Vettorazzi et al., 2017) but also in several other plant species including *Bacopa monnieri* (Behera et al., 2015), *Punica granatum* (Naik et al., 1999, Patil et al., 2011), and *Symplocos racemosa* (Behera et al., 2017). Nodal segments has also been used for micropropagation of other *Ipomoea* species as well i.e., *I. mauritiana* (Islam and Bari, 2013), and *I. batatas* (Beyene et al., 2020; Dewir et al., 2020; Karan and Ozdemir, 2021).

MS medium was used as basal medium for this *in vitro* plant regeneration study, MS medium is the most commonly used basal medium for plant tissue culture of not only sweet potato (Liao and Chung, 1979; Kuo et al., 1985; Beetham and Mason, 1992; Mukherjee, 2002; Gong et al., 2005; Yang, 2010; Dolinski and Olek, 2013; Alula et al., 2018; Abubakar et al., 2018; Mengs et al., 2018; Arif and Bahari, 2019) but also in several plant species (Naik and Chand, 2011). But only MS basal medium devoid of PGRs was not sufficient for multiple shoot proliferation. Therefore, several types, concentrations and amalgamations of cytokinins and auxins were tried to find out the optimum growth regulator supplement to MS medium for optimum shoot proliferation from nodal segments. MS basal medium fortified with mT 2.0 mg/l was the most effective cytokinin for shoot multiplication from nodal explants when compared to all other cytokinins and combination of cytokinins and auxins tested. First isolated from poplar leaves and subsequently isolated from Populus × Canadensis Moench cv. Robusta, mT is an aromatic cytokinin (Strnad et al., 1997; Behera et al., 2019). Many reports documented optimum multiple shoot proliferation using mT along with MS basal medium which includes plants such as *Barleria sorbustorminalis* (Mala et al., 2009), *Prunus* stock (Gentile et al., 2014), *Hedychium coronarium* (Behera et al., 2019), *Scaevola taccada* (Shekhawat et al., 2021), *Vanilla planifolia* (Manokari et al., 2021a), *Santalum album* (Manokari et al., 2021b), *Dioscorea pentaphylla* (Manokari et al., 2022), *Crinum malabaricum* (Chahal et al., 2022), and *Curcuma amada* (Behera et al., 2022b). In this study, it was observed that mT was more efficient for shoot multiplication and elongation of purple flesh sweet potato var. 'Bhu Krishna' than BA and other cytokinins may be due to its less toxicity (Behera et al., 2022a). Furthermore, the structural and functional advantage of mT over BA is due to the existence of hydroxyl group in its side chain that leads to the formation and accumulation of *O*-glucoside (Werbrouck et al., 1996; Ahmad and Anis, 2019; Behera et al., 2022b). Based on necessities, these *O*-glucosides are easily altered into energetic nucleotides, nucleosides and/or free bases and helps in multiple shoot formation and elongation (Strnad et al., 1997; Behera et al., 2019). To enhance the production of shoot number, *axenic* nodal explants were excised from shoot culture and further cultured on optimum shoot multiplication medium and approximately 1300 shoots were generated from each nodal explant in twelve weeks.

In earlier micropropagation reports of sweet potato, the rooting of *axenic* shoots has been reported on growth regulator free MS medium (Templeton-Somers and Collins, 1986; Sonnino and Mini, 1993; Mukherjee, 2002), or on $^1/_2$ MS medium (Schultheis and Cantliffe, 1992; Dugassa and Feyissa, 2010). However, in this study $^1/_2$ MS with IBA (0.5 mg/l) was found to be the optimum medium for rooting of *axenic* shoots. This result concurs with the earlier study on sweet potato by Dugassa and Fevissa (2010), where $^1/_2$ MS medium supplemented with IBA has been used for rooting in sweet potato var. Awassa-83 and Awassa local. Various potting substrates either alone or in combinations have been used for acclimatization and field establishment of sweet potato with different survival rate. Micropropagated plants were acclimatized in the potting tray containing coco peat, soil, and sand (1:1:1) and subsequently transferred to larger clay pots and then established in the field with 95% of survival rate. Whereas, Oggero et al., (2011) reported acclimatization of micropropagated plants of sweet potato var. 'KEMB 36' in the pot containing rice husks and red soil (1:2) and about 80% of plantlets were successfully established. Oggero et al. (2012) also acclimatized two cultivars of sweet potato (Kemb-36 and Tainurey) on the same potting substrate rice husk and red soil and found that the survival rate is comparatively less i.e. 67%. A mixture of sterilized potting soil, vermiculite and sand (1:1:1) has been used as a potting substrate by Gong et al. (2005) and in this potting substrate, plants (sweet potato cv. Gaozi No. 1) were survived successfully. Ubalua et al. (2013) has reported that Jiffy peat substrate is the most superior substrate for cent-percent acclimatization of micropropagated sweet potato var. King J.

4.2. Assessment of genetic fidelity and biochemical analysis

In plant biotechnology, the plant cell, tissue, and organ culture has been accepted as a promising tool for large scale production of genetically and biochemically uniform plants (Behera et al., 2018; Sahu and Panigrahi, 2018). However, there is a possibility of developing somaclonal variations due to various factors, such as culture conditions, types and concentrations of PGRs, etc. (Sahu and Panigrahi, 2018). Thus, evaluation of genetic fidelity is very essential in tissue culture raised plants. Assessment of genetic uniformity of such plants using PCR based markers has an advantage as it validate the clonal propagation protocol that is being developed for production of large scale true-to-types plants for commercial utilization. Thus, micropropagated plants of purple flesh sweet potato var. 'Bhu Krishna' was subjected to molecular analysis to assess their genetic stability in comparison with the mother plant. Several PCR based molecular markers had been used to evaluate the clonal stability of micropropagated plants by different authors. However, ISSR primer is the cheapest yet reliable, simple, requires a small quantity of DNA, and does not require a prior genome sequence for amplification (Behera et al., 2018; Sahu and Panigrahi, 2018; Behera et al., 2019). In this study, ISSR primer was used to assess the genetic fidelity of micropropagated plants and found that all ISSR primers amplified uniform bands, indicating micropropagated plants were genetically stable with the mother plant. This marker has also been used in many micropropagated plants to assess their genetic fidelity including, *Bacopa monnieri* (Pramanik et al., 2021), *Globba marantina* (Parida et al., 2018), *Hedychium coronarium* (Behera et al., 2019; Behera et al., 2023a), and *Scaevola taccada* (Shekhawat et al., 2021).

Similar to genetic uniformity, biochemical analysis of micropropagated plants is also essential. Thus, before releasing micropropagated plants to the market or supply to the farmers or industrial utilization, their biochemical fidelity needs to be checked. Keeping this point in view, the biochemical analysis in terms of alkaloids, anthocyanin,

flavonoids, phenolics, saponins, sugar, starch, and tannins content of micropropagated plants and the mother plant was carried out. This study revealed that micropropagated plants were phytochemically stable with that of the mother plant. 'Bhu Krishna' is rich in anthocyanin, which is significantly higher than other Sweet potato varieties, including and similar to some crops which are rich in anthocyanin content like blueberries, blackberries, cranberries as well as grapes (Li et al., 2019). The purple flesh Sweet potatoes rich in anthocyanin have been extensively used as a food additive in several countries in the globe, including India and China (Fan et al., 2008; Li et al., 2019).

4.3. Anticancer property study

Plants are rich in several important secondary metabolites of therapeutic values (Behera et al., 2023b). Secondary metabolites are known to have the ability to alleviate different chronic diseases, including cancer, atherosclerosis, ageing, vision disorder, diabetes, hypertension, neurodegenerative, and liver dysfunction (Sugata et al., 2015). Recently, plant secondary metabolites have gained huge attention from researchers, pharmaceutical industries, and cosumers due to its low side effect. Till date, a number of anticancer drugs have been developed using plant secondary metabolites including taxol, doctaxel, navelbine, vincristine, vinblastine, eldisine, and cabazitaxel and their biological activity and clinical application have also proven in cancer treatment (Shasmita et al., 2018; Behera et al., 2023b).

Purple flesh sweet potato is rich in anthocyanins and has antioxidant, anti-inflammatory, and anticancer properties (Zhang et al., 2009; Zhao et al., 2013; Xu et al., 2018; Tang et al., 2023). Thus, purple flesh sweet potato have received increasing attention. Till date, a number of reports have documented anticancer activity of tuber extract or bioactive compounds isolated from various purple flesh sweet potato against different cancer cell lines. i.e., human colon cancer cell lines (SW80, WiDr), breast cancer cell lines (MCF-7), gastric cancer cell lines (SNU-1), prostate cancer cell lines (PC-3, C4-2, C-2B, DU145, and LNCaP), and bladder cancer cell lines (5637 and T24) (Lim et al., 2013; Sugata et al., 2015; Silva-Correa et al., 2022; Tang et al., 2023). Purple flesh sweet potato var. Bhu Krishna is also rich in anthocyanin. Therefore, in this study, the anticancer property of the tuber samples of both the mother plant and the micropropagated plants was conducted against two different breast cancer cell lines i.e., MCF-7 and MDA-MB-231 to confirm the retention of the therapeutic activity of the tuber of tissue cultured sweet potato. It was found that therapeutic activity of the micropropagated plants was comparable to the mother plant. This sweet potato variety 'Bhu Krishna' showed promising anticancer activities may be due to the presence of anthocyanin. Recent reports suggested that anthocyanin significantly reduced the cancer cell viability by decreasing mitochondrial membrane potential and increasing intracellular reactive oxygen species production leding to cancer cell apoptosis (Tang et al., 2023). The cancer cell apoptosis induces due to the activation of both the mitochondrial Bax/Cytochrome-c/Caspase-9/Caspase-3 pathway and extrinsic Fas/Caspase-8/Caspase-3 pathway (Sugata et al., 2015). Whereas, Guo et al. (2021) reported that Cyclin B1 induced the progression of cell from G2 phase to M phase and probably sweet potato anthocyanin downregulates the Cyclin B1 expression, which leads to cell apoptosis. In this study, a protocol has been developed to produce genetically uniform, biochemically and therapeutically stable micropropagated plants of anthocyanin-rich sweet potato var. 'Bhu Krishna'.

5. Conclusion

Purple flesh sweet potato var. 'Bhu Krishna' is having high food and industrial value as well as medicinal importance due to rich in anthocyanin. Here a highly efficient micropropagation protocol for the variety 'Bhu Krishna' using mature nodal segments has been developed for the first time. The plants produced using this protocol will be useful for the production large number of healthy, clonally stable (genetically and biochemically) plants around the year to meet the demand of farmers for good planting materials as well as industrial uses.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Author contributions

SB designed the experiment, carried out investigation, and wrote the paper. SKK helped SB in the genetic fidelity experiment. KM helped SB in biochemical analysis. RKM helped SB in the anticancer activity study. SB conducted all data analysis. VBSC and SKN supervised the tissue culture work and data analysis. KP, VVB, MN, PKN, AKV and SKN edited the manuscript. All authors read, corrected, and approved the final draft for publication.

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