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ORIGINAL ARTICLE

Plant growth regulators from microalgae biomass and their impact on the genetic fidelity of canola and tomato plantlets

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Abstract

The present study aimed to evaluate the genetic divergence of canola and tomato in vitro plantlets cultured on MS medium supplemented with the plant growth regulators produced from some algae cultivated on $BG11_0$ for Cyanobacteria species (*Anabaena oryzae* and *Nostoc muscorum*) and BG11 for Chlorophyta species (*Chlorella vulgaris*) (as control media), grown on treated sewage wastewater concentration (TSW combined with control media) (100, 75, and 50%) and different concentrations of nitrogen (0, 0.5, 2, and 4 of $NaNO_3$ in BG11) for Chlorophyta. The experiment was conducted in triplicate, and cultures were incubated at 25 ± 1 °C under continuous aeration (1.25 L/min), 16:8 h light and dark cycle and illumination (40 µE/m^2/s), and harvested at the end of the exponential phase. The HPLC was used for growth regulator (auxins and cytokinins) analysis in different algal treatments. Apical buds of canola and tomato seedlings were dissected and transferred to MS media with algal extract containing the optimum concentration of benzyl adenine (BA) and indole acetic acid (IAA) and different concentrations of synthetic hormones.Basal solidified MS medium and/or dimethyl sulfoxide (DMSO) were considered as control. Genetic stability among the produced in vitro plantlets of both plant species was evaluated by inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) markers. The whole banding pattern of in vitro plantlets of both species revealed their low divergence compared to controls when cultured on medium with algal-based plant hormones. This work indicated that the use of sewage wastewater as media for cultivation of the investigated algae is safe, suitable, cheap, and not expensive methods when compared with the ordinary used media.

Keywords Chlorophyta · Cyanobacteria · ISSR · In vitro plantlets · RAPD · Sewage wastewater

1 Introduction

At present, our knowledge of the algal hormonal system is still rather fragmentary. Until now, the presence of the full-value hormonal system in algae and the correspondence of their biological activities to those of higher plant hormones are debated.

Treated sewage waste waters (TSW) were largely available and used as cheap nutritive culture media for microalgal growth and large-scale biomass production. They contain the essential elements, macro- and micronutrients needed for algal cultivation [1–4].

Many studies highlighted the importance of micro- and macro-algal filtrates as well as cyanobacterial extracts either added to the soil or mixed with the tissue culture media or even applied as foliar spray 5, 15, 17, 21). Also, the effect of seaweed extracts made from the brown algae as *Durvillaea potatorum* and *Ascophyllum nodosum* on tomato plant and soil was shown to be highly beneficial [5–8]. In addition, these extracts also enhanced the morphological, physiological, and biochemical parameters of different crop plants and vegetables [9–11].

Algal extracts were proved to contain growth-promoting substances (phytohormones) and other bio-stimulants as amino acids, vitamins, and macro- and micronutrients, which induced stimulatory effects to plant growth [12] by enhancing water uptake, root and shoot growth, and tolerance to stresses [8] on all developmental stages as germination of seeds and growth of plant seedlings till plant harvest.

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Lower algal extract concentrations were found to be more effective either applied for seed germination or seedling growth [13, 14].

The worldwide importance of plant biotechnology may be due to its increased sustainability, which is a beneficial application in agriculture [15]. One of the most commercially exploited roles of biotechnology has been the search of cheap and safe bio-fertilizers that act similarly to the synthetic ones but do not affect the genetic makeup of the cultivated crops so entrepreneurs can ultimately use them without any ambiguity [16–18].

The occurrence of genetic variation is a great threat where commercial success in micropropagation depends only on the preservation of clonal uniformity [19]. In most cases, micropropagated plants derived through meristems' culture were reported to retain their genetic fidelity. The capacity to analyze those variations was crucial and need the use of molecular DNA markers such as inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), and many others [20]. Largia et al. [21] used 25 RAPD and 20 ISSR primers to analyze the genetic similarity of in vitro-regenerated plants derived from encapsulated shoot tips of *B. monnieri* using PGRs.

This work aimed to evaluate the laboratory cultivation of microalgae on secondary treated sewage wastewater (TSW) and different nitrogen concentrations for biomass and production of plant growth regulators (IAA, BA) as well as to determine bio-fertilization properties of the promising extracts and the genetic fidelity of in vitro plantlets (by ISSR and RAPD markers) affected by them.

2 Materials and methods

2.1 Plant material

Canola seeds (*Brassica napus L.)* were obtained from the Agriculture Research Center, Giza, Egypt. Tomato seeds (*Lycopersicon esculentus* L. moneymaker) were purchased from the US supermarket.

2.1.1 Algal samples

Algal species and culture conditions Cyanobacterial strains (*Anabaena oryzae* and *Nostoc muscorum*) and Chlorophyta strain (*Chlorella vulgaris*) were obtained from the Microbiology Department, Soils, Water and Environment Res. Inst. (SWERI), Agric. Res., Center (ARC). The algae were cultured and maintained on liquid $BG11_0$ free nitrogen [22] for cyanobacteria and BG11 media [23] for green algae. Cultures were incubated in a growth chamber under continuous aeration (1.25 L/min), 16:8 h light and dark cycle and light intensity (40 µE/m^2/s) at 25 ± 1 °C, and harvested at the end of the exponential phase,

2.2 Wastewater sources

Sewage wastewater (SWW) was obtained from Zenain station, Giza, Egypt.

2.3 Wastewater analysis

Chemical and physical parameters of different wastewater concentrations were analyzed as reported by APHA [24] (Table 1).

2.4 Treatment with sewage wastewater and different nitrogen ($NaNO_3$) concentrations

The SWW was sterilized using glass microfiber filter (0.22 µm) to remove large particles and indigenous bacteria for the experiment; this was signed as treated sewage wastewater (TSW) Wang et al. [4]. TSW was applied separately and in combination with $BG11_0$ and BG11 to study their effects on growth of algal strains, as well as nitrogen different concentrations (0, 0.5, 2, and 4 × of $NaNO_3$) for *C. vulgaris*. $BG11_0$ and BG11 media were used as control media

Table 1 Physical and chemical analysis of the treated sewage wastewater (TSW) used for the cultivation of algae compared with BG11 medium composition

Parameters	BG11 medium	Treated sewage wastewater (TSW)
pH	7	6.48
Total nitrogen mg/L	248	28.5
Cations mg/L		
Ca^{2+}	9.8	36.7
Mg^{2+}	8.8	16.2
K^+	17.9	17.5
Na^+	140	177
Anions mg/L		
CO_3^{2-}	6.2	39.9
HCO^{3-}	13	183
Cl^-	18	71
SO_4^{2-}	37	263
Trace elements (µg/L)		
Cu	20	36
Zn	5	62
B	500	150
P	7000	6100
Fe	3200	130
Co	16	27
Mn	500	110

representing the standard synthetic media. The experiment was conducted as we reported in our previous investigation [25].

2.5 PGR extraction

The algal cells were harvested in each experiment at the end of the exponential phase, centrifuged, oven dried (50 °C for 24 h), and extracted overnight in 96% methanol according to the method of Sun et al. [26]. Then, the methanolic fraction was filtrated, and the residual pellets were re-extracted 3 times with 40% (10 ml) cold methanol. The combined methanol extracts were evaporated in the dark at room temperature. The residual aqueous solution was adjusted to pH 2.6–2.8 and then extracted 3 times by absolute ethyl acetate (50 ml/each extract). In addition, the ethyl acetate fraction was separated and dried over anhydrous MgSO4. Finally, the residue was dissolved in 4 ml of absolute methanol.

2.6 HPLC conditions

The standard hormonal samples as well as the algal methanol extracts were subjected successively to be analyzed by high-performance liquid chromatography (HPLC). The quantitative analysis of phytohormones was performed with YL9100 HPLC system with C_{18} column, UV at 254 nm and 40 ºC with gradient mode of methanol and acetic acid. The amount of IAA and BA in the alga grown on $BG11_0$ or BG11 alone and combinations with TSW as well as nitrogen different concentrations of *C. vulgaris* was calculated from the dose growth curves and the HPLC analysis of algal hormones.

2.7 Seed sterilization

The seeds of tomato and canola were surface sterilized by immersing in 30% Clorox® (5.25% w/v sodium hypochlorite) for 30 min with stirring and then washed several times by sterile distilled water. The seeds were transferred to 100 ml flasks containing 70% ethanol, with stirring for 2 min. The alcohol was decanted, and then the seeds were washed thoroughly (5 times) with sterile double-distilled water under a sterile air of laminar flow hood [27].

2.8 Seed germination

All seeds (tomato and canola) were germinated separately in vitro on basal Murashige and Skoog medium (MS) [28] supplemented with 30 g/L sucrose and 100 mg/L myo-inositol and solidified with 0.8% agar. The jars were incubated in growth room with constant temperature (25 ± 0.2 ºC) and photoperiod (16 h dark/8 h light).

2.9 Seedling of canola and tomato

Aseptically grown canola and tomato seedlings (14-day-old) were used as a source of plantlets.

2.10 Experiment of canola and tomato seedlings

Apical buds of both seedlings were cut and transferred to MS media with different additives: algal sample extracts at the optimum concentration of BA and IAA (calculated from their dose–response curves and HPLC analysis) and different concentrations of synthetic hormones.Basal solidified MS medium was considered as control as well as MS medium with dimethyl sulfoxide (DMSO) (used as solvent). For both seedlings, each treatment was represented by triplicate jars, and each jar contains 4 seedlings. All jars were incubated in growth room at constant temperature (25 ± 0.2 ºC) and 16 h dark/8 h light photoperiod for 10 days. All in vitro plantlets were subjected to molecular analyses.

2.11 Molecular analyses

Whole genomic DNA was extracted from 50 mg of liquid nitrogen powdered plantlets (triplicates) collected from each tomato and canola plantlets (treated and control) using CTAB extraction protocol of Doyle and Doyle [29] with minor modifications.

PCR amplifications were carried out using ISSR and RAPD primers chosen according to their ability to produce clear banding pattern (Table 20). The reaction mixture comprised 25 µl containing 12.5 µl Thermo Scientific DreamTaq™ Green PCR Master Mix (2×), 2 µl primer, and 1 µl (50 ng) template DNA. For ISSR, the amplification was performed using Veriti™ 96-*Well Thermal Cycler*as follows: initial denaturation of 5 min at 95 °C; 40 cycles of 30 s denaturation at 95 °C, 30-s annealing at 56 °C and 2 min extension at 72 °C, and a final elongation step at 72 °C for 10 min. In case of RAPD, the amplification was conducted through initial denaturation of 5 min at 92 °C; 40 cycles of 30 s denaturation at 92 °C, 1 min annealing at 35°C, ramp up for 5 min at 72 °C and 2 min extension at 72 °C, and a final extension at 72 °C for 10 min.

PCR products were separated by electrophoresis (3 h, 80 v) through 1 % (m/v) agarose gel in 1× TBE. Band sizes were visualized and determined using gel documentation (G:BOX) (SYNGENE model 680XHR, UK) based on 3000 by DNA ladder.

2.12 Statistical analysis

Data were subjected to an analysis of variance, and the means were compared using the least significant difference (LSD) test at the 0.05 levels ($p \leq 0.05$), as recommended by Snedecor and Cochran [30].

3 Results and discussion

3.1 HPLC results

From our previous investigation, we found that the plant growth regulators, especially IAA and BA, were produced from some algae cultivated on $BG11_0$ for Cyanobacteria species (*Anabaena oryzae* and *Nostoc muscorum)*, BG11 for Chlorophyta species (*Chlorella vulgaris*) (as control media), treated with sewage wastewater concentration (TSW combined with control media) (100, 75 and 50%), and different concentrations of nitrogen (0, 0.5, 2, and 4 of $NaNO_3$ in BG11) for Chlorophyta. The analysis of plant growth regulators especially IAA and BA in different extracts of cultivated *Chlorella vulgaris* revealed that TSW 100% had the highest concentration of IAA (0.032 mg/g) and BA (0.695 mg/g) and at $4 \times NaNo_3$ concentration medium and IAA (0.007 mg/g) and BA (0.005 mg/g) when compared with BG11 medium (0.01 mg/g IAA and 0.004 mg/g BA). In case of *Anabaena oryzae*, the highest concentration of IAA (0.051 mg/g) was found on TSW 100% when compared with $BG11_0$ medium (0.003 mg/g). The highest BA (0.044 mg/g) was recorded when the alga was cultivated in 50% TSW as compared with $BG11_0$ medium (0.013 mg/g). The analyses of IAA and BA in different extracts of *Nostoc muscurum* cultivated in TSW and $BG11_0$ media revealed that TSW 100% recorded the highest concentration of IAA (0.053 mg/g) and BA (0.120 mg/g) when compared with $BG11_0$ medium (0.002 mg/g IAA and 0.014 mg/g BA) (Tables 2 and 3). From those results, we cultured the three algae on 100% TSW to obtain the highest values of both PGRs and used them as start material for the following experiments.

3.2 Canola experiment

The application experiment used various auxin (IAA) and cytokinin (BA) concentrations separately and in combination as well as tested samples of optimum concentration and the effect of its crude extract obtained from cultures on various media on the morphological parameters of canola plantlets. MS and DMSO media represented the controls. The effect was focused on morphological parameters (shoot length, leaves' numbers, leaf expansion, root initiation, and branching) (Fig. 1).

Table 4 of the obtained results of *A. oryzae* showed that indole acetic acid (IAA) at a concentration of 1.8 ppm recorded the greatest shoot length (6.600 ± 1.147) compared to MS and DMSO media and other IAA concentration (0.5, 2.5 ppm) as well as the combination IAA + BA concentration. The shoot had moderate leaves' number (3.333 ± 0.651) and greatest root initiation, while no branching and no leaf expansion were recorded. Both lower IAA concentration

Table 2 HPLC analysis of PGRs (mg/g) extracted from cyanobacterial and Chlorophyta species cultivated on $BG11_0$ or BG11 (as a control) and TSW media

Sample crude extracts	Hormones	R.T. min	100% TSW		75%TSW		50%TSW		Control medium	
			Conc. mg/g	R%	Conc. mg/g	R%	Conc. mg/g	R%	Conc. mg/g	R%
A. oryzae	IAA	9.4	0.051	67.5	0.012	38	0.011	17.7	0.003	66.7
	BA	10.5	0.023	32.5	0.021	62	0.044	82.3	0.013	33.3
N. muscorum	IAA	9.4	0.053	31.5	0.025	88.6	0.050	67.5	0.002	14.49
	BA	10.5	0.120	68.5	0.004	11.4	0.017	32.5	0.014	85.51
C. vulgaris	IAA	9.4	0.032	4.4	0.005	70.2	0.014	28.5	0.01	75.3
	BA	10.5	0.695	95.7	0.005	29.8	0.033	71.5	0.004	24.7

TSW, treated sewage wastewater; *IAA*, indole acetic acid; *BA*, benzyl adenine

Table 3 HPLC analysis of PGRs (mg/g) extracted from *Chlorella vulgaris* cultivated in different concentrations of nitrogen ($NaNO_{3)}$ media

Hormones	R.T. min	0×N		0.5×N		2×N		4×N		BG11	
		Conc. mg/g	R%	Conc. mg/g	R%	Conc. mg/g	R%	Conc. mg/g	R%	Conc. mg/g	R%
IAA	9.4	0.005	44.1	0.006	46.3	0.008	80.8	0.007	55.9	0.01	75.26
BA	10.5	0.005	55.9	0.006	53.7	0.002	19.2	0.005	44.1	0.004	24.74

x, conc.; *N*, nitrogen ($NaNO_3$); *IAA*, indole acetic acid; *BA*, benzyl adenine

Fig. 1 Growth of canola explants by synthetic BA and IAA and mixtures of IAA + BA concentration as well as sample and extracts (grown on various media) with optimum concentration of BA and IAA (from dose response curve) on canola plant

(0.5 ppm) and higher concentration (2.5 ppm) were less effective concerning shoot length and leaves' number. Morphological parameters of canola explants were enhanced by moderate concentration of exogenous hormones (IAA + BA, 1.8 + 2.5 ppm). Comparable results were recorded by *A. oryzae* extract cultivated on $BG11_0$ contained 0.5 + 10 ppm. Higher IAA + BA mixture concentration (2.5 + 50 ppm) showed lesser stimulatory effect to all parameters except root initiation which was largely inhibited. *A. oryzae* extracts (grown on $BG11_0$ and 100% TSW media) showed moderate shoot length and leaves' number of canola explant compared to control, DMSO, and IAA concentration as well as IAA + BA concentration which were shown to be without any effect on root initiation, branching, and leaf expansion, The crude extract of *A. oryzae* with optimum concentration of BA had the same trend, as shown in Table 5.

The obtained results of *N. muscurum* were shown in Table 6 with the tested *N. muscorum* optimum concentration

Table 4 The effect of *Anabaena oryzae* crude extracts (grown on BG-11_0 and 100% TSW) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on canola plant

Parameter	Shoot length cm	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.858 ± 1.058^{ab}	3.583 ± 0.996^{ab}	+	+ +	-
DMSO	5.483 ± 1.192^{ab}	2.750 ± 0.754^{ab}	-	-	-
IAA (0.5 ppm)	5.617 ± 1.446^{ab}	3.333 ± 0.651^{ab}	-	-	-
IAA (1.8 ppm)	6.600 ± 1.147^{a}	3.333 ± 0.651^{ab}	+ + +	-	-
IAA (2.5 ppm)	4.800 ± 1.059^{b}	2.917 ± 0793^{ab}	-	-	-
IAA + BA (0.5 + 10) ppm	4.858 ± 0.897^{b}	2.667 ± 0.778^{b}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{ab}	3.667 ± 1.614^{a}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	5.958 ± 1.593^{ab}	3.083 ± 0.793^{ab}	-	+	+ With long petiole
A. oryzae ext. ($BG11_0$)	4.725 ± 0.561^{b}	2.667 ± 0.778^{ab}	-	-	-
A. oryzae ext. (100% TSW)	5.708 ± 0.406^{ab}	2.667 ± 0.651^{ab}	-	-	-

(-) = absence; (+ + +) = great effect; (+ +) = moderate effect; (+) = lowest effect; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *IAA*, indole acetic acid; *BA*, benzyl adenine; *TSW*, treated sewage wastewater

Table 5 The effect of *Anabaena oryzae* crude extracts (grown on BG-11_0 and 100% TSW) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on canola plant

Parameter	Shoot length cm	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	$5.858 \pm 1.058^{a\,b}$	3.583 ± 0.996^{ab}	+	+ +	-
DMSO	5.083 ± 0.925^{abc}	2.750 ± 0.754^{b}	-	-	-
BA (10 ppm)	4.342 ± 0.558^{c}	3.083 ± 0.669^{b}	-	+	+
BA (25 ppm)	5.533 ± 1.608^{abc}	4.333 ± 1.073^{a}	-	+ + +	+ + + With long petiole
BA (50 ppm)	6.125 ± 1.869^{a}	4.333 ± 1.073^{a}	-	+ + +	+ + + With long petiole
IAA + BA (0.5 + 10) ppm	$4.817 \pm 0.926^{a\,b}$	2.667 ± 0.778^{b}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{abc}	3.667 ± 1.614^{ab}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	$5.958 \pm 1.593^{a\,b}$	3.083 ± 0.793^{b}	-	+	+ With long petiole
A. oryzae ext. ($BG-11_0$)	$4.767 \pm 0.460^{a\,b}$	2.750 ± 0.622^{b}	-	-	-
A.oryzae ext. (100% TSW)	4.908 ± 0.498^{b}	2.917 ± 0.669^{b}	-	-	-

(-) = absence; (+ + +) = great effect; (+ +) = moderate effect; (+) = lowest effect; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *TSW*, treated sewage wastewater

on $BG11_0$ and 100% TSW media revealed that 100% TSW recorded the greatest shoot length (7.117 ± 1.243) compared to MS and DMSO media and other IAA concentrations (0.5, 2.5, and 1.8 ppm) as well as the combination IAA + BA concentration. The shoot had moderate leaves' number (3.500 ± 0.522) and less root initiation, while no branching and no leaf expansion were recorded. *N. muscurum* extract (grown on $BG11_0$ medium) showed moderate shoot length and leaves' number of canola explant compared to control, DMSO, and IAA concentration as well as IAA + BA concentration without any effect on root initiation, branching, and leaf expansion; 1.8 ppm IAA and 1.8 + 25 ppm IAA + BA induced great root initiation, branching, and leaf expansion which occurred at higher concentration of IAA and BA (1.8 + 25/2.5 + 50 ppm). The crude extract of *N. muscurum* with optimum concentration of BA had the same trend, as shown in Table 7 especially when BA concentration increased either singly or combined with IAA.

Table 8 illustrated the obtained results of *C. vulgaris* and showed that IAA at concentration 1.8 ppm and 100% TSW recorded the greatest shoot length (6.600 ± 1.147, 6.583 ± 0.859) compared to MS and DMSO media and other IAA concentration (0.5, 2.5 ppm) as well as the combination IAA + BA concentration. The shoot had moderate leaves' number (3.333 ± 0.651, 3.417 ± 0.793) and the greatest root initiation, while no branching and no leaf expansion were recorded at 1.8 ppm IAA and 100% TSW, while at 4 × nitrogen concentration, a great shoot length (6.033 ± 1.231), leaves' number (3.417 ± 0.793), and lower root initiation were recorded. Both lower

Table 6 The effect of *Nostoc muscorum* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on canola plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.858 ± 1.058^{abc}	3.583 ± 0.996^{ab}	+	+ +	-
DMSO	5.483 ± 1.192^{bc}	2.750 ± 0.754^{ab}	-	-	-
IAA (0.5 ppm)	5.617 ± 1.446^{bc}	3.333 ± 0.651^{ab}	-	-	-
IAA (1.8 ppm)	6.600 ± 1.147^{ab}	3.333 ± 0.651^{ab}	+ + +	-	-
IAA (2.5 ppm)	4.800 ± 1.059^{c}	2.917 ± 0793^{ab}	-	-	-
IAA + BA (0.5 + 10) ppm	4.858 ± 0.897^{c}	2.667 ± 0.778^{b}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{bc}	3.667 ± 1.614^{a}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	5.958 ± 1.593^{abc}	3.083 ± 0.793^{ab}	_	+	+ With long petiole
N. muscorum ($BG11_0$)	5.025 ± 0.618^{c}	3.250 ± 0.754^{ab}	-	-	-
N. muscorum (100%TSW)	7.117 ± 1.243^{a}	3.500 ± 0.522^{ab}	+	-	-

(-) = absence; (+ + +) = great effect; (+ +) = moderate effect; (+) = lowest effect; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *TSW*, treated sewage wastewater

Table 7 The effect of *Nostoc muscorum* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on canola plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.858 ± 1.058^{a}	3.583 ± 0.996^{ab}	+	+ +	-
DMSO	5.492 ± 1.200^{ab}	2.750 ± 0.754^{b}	-	-	-
BA (10 ppm)	4.342 ± 0.558^{b}	3.083 ± 0.669^{b}	-	+	+
BA (25 ppm)	5.533 ± 1.608^{ab}	4.333 ± 1.073^{a}	_	+ + +	+ + + With long petiole
BA (50 ppm)	6.125 ± 1.869^{a}	4.333 ± 1.073^{a}	_	+ + +	+ + + With long petiole
IAA + BA (0.5 + 10) ppm	4.817 ± 0.926^{ab}	2.667 ± 0.778^{b}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{ab}	3.667 ± 1.614^{ab}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	5.958 ± 1.593^{a}	3.083 ± 0.793^{b}	_	+	+ With long petiole
N. muscorum ($BG11_0$)	4.758 ± 0.507^{ab}	3.083 ± 0.669^{b}	-	+	-
N. muscorum (100% TSW)	6.133 ± 0.838^{a}	3.667 ± 0.492^{ab}	-	-	-

(-) = absence; (+ + +) = great effect; (+ +) = moderate effect; (+) = lowest effect; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *TSW*, treated sewage wastewater

IAA concentration (0.5 ppm) and higher concentration (2.5 ppm) were less effective concerning shoot length and leaves' number. Morphological parameters of canola explants were enhanced by moderate concentration of exogenous hormones (IAA + BA, 1.8 + 2.5 ppm). Comparable results were recorded by *C. vulgaris* extract cultivated on BG11 (contained 0.5 + 10 ppm). The crude extract of *C. vulgaris* with optimum concentration of BA, 100% TSW, and 4 × N concentration had the greatest shoot length and moderate leaves' number, while no root initiation, branching, and leaf expansion was induced, as shown in Table 9.

3.3 Tomato experiment

This application experiment also used various auxin (IAA) and cytokinin (BA) concentrations separately and in combination as well as tested samples with optimum concentration and the effect of its crude extract obtained from cultures on various media on the morphological parameters of tomato plantlets. MS and DMSO media represented the controls. The effect was focused on morphological parameters (shoot length, leaves' numbers, leaf expansion, root initiation, and branching) (Fig. 2).

Table 8 The effect of *Chlorella vulgaris* crude extracts (Grown on BG11, 100% TSW and 4×N media) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on canola plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.858 ± 1.058^{ab}	3.583 ± 0.996^{a}	+	+ +	-
DMSO	5.483 ± 1.192^{ab}	2.750 ± 0.754^{ab}	-	-	-
IAA (0.5 ppm)	5.617 ± 1.446^{ab}	3.333 ± 0.651^{ab}	-	-	-
IAA (1.8 ppm)	6.600 ± 1.147^{a}	3.333 ± 0.651^{ab}	+ + +	-	-
IAA (2.5 ppm)	4.800 ± 1.059^{b}	2.917 ± 0793^{ab}	-	-	-
IAA + BA (0.5 + 10) ppm	4.858 ± 0.897^{b}	2.667 ± 0.778^{ab}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{ab}	3.667 ± 1.614^{a}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	5.958 ± 1.593^{ab}	3.083 ± 0.793^{ab}	-	+	+ With long petiole
C. vulgaris (BG-11)	5.142 ± 0.653^{b}	2.500 ± 0.522^{b}	-	-	-
C. vulgaris (100% TSW)	6.583 ± 0.859^{a}	3.417 ± 0.793^{ab}	+ +	-	-
C. vulgaris (4×N)	6.033 ± 1.231^{ab}	3.167 ± 0.835^{ab}	+	-	-

(-) = absence; (3 +) = great effect; (2 +) = moderate effect; (+) = lowest effect; *4×N*, BG11medium with fourfold of $NaNO_3$ concentration; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *TSW*, treated sewage wastewater

Table 9 The effect of *Chlorella vulgaris* crude extracts (grown on BG11, 100% TSW, and 4×N media) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on canola plant

Parameter	Shoot length cm	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.858 ± 1.058^{ab}	3.583 ± 0.996^{abc}	+	+ +	-
DMSO	5.492 ± 1.200^{abc}	2.750 ± 0.754^{c}	-	-	-
BA (10 ppm)	4.342 ± 0.558^{c}	3.083 ± 0.669^{c}	-	+	+
BA (25 ppm)	5.533 ± 1.608^{abc}	4.333 ± 1.073^{ab}	_	+ + +	+ + + With long petiole
BA (50 ppm)	6.125 ± 1.869^{ab}	4.333 ± 1.073^{a}	_	+ + +	+ + + With long petiole
IAA + BA (0.5 + 10) ppm	4.817 ± 0.926^{bc}	2.667 ± 0.778^{c}	-	-	-
IAA + BA (1.8 + 25) ppm	5.508 ± 1.045^{abc}	3.667 ± 1.614^{abc}	+ + +	+ +	+
IAA + BA (2.5 + 50) ppm	5.958 ± 1.593^{ab}	3.083 ± 0.793^{c}	-	+	+ With long petiole
C. vulgaris (BG11)	5.717 ± 0.587^{abc}	2.917 ± 0.793^{c}	-	-	-
C. vulgaris (100%TSW)	6.675 ± 1.123^{a}	3.417 ± 0.515^{abc}	-	-	-
C. vulgaris (4×N)	6.300 ± 0.506^{a}	3.333 ± 0.492^{bc}	-	-	-

(-) = absence; (3 +) = great effect; (2 +) = moderate effect; (+) = lowest effect; *4×N*, BG11medium with fourfold of $NaNO_3$ concentration; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium; *TSW*, treated sewage wastewater

Table 10 of the obtained results of *A. oryzae* showed that IAA at concentration 1.8 ppm recorded the greatest shoot length (6.850 ± 1.588) compared to MS and DMSO media and other IAA concentrations (0.5, 2.5 ppm) as well as the combination IAA + BA concentration. The shoot had high leaves' number (3.583 ± 0.515) and the greatest root initiation, while no branching and no leaf expansion were recorded. The lower IAA concentration (0.5 ppm) was less effective concerning shoot length and leaves' number but with high root initiation (6 +), while higher concentration (2.5 ppm) has high efficiency concerning leaves' number and shoot length. Morphological parameters of tomato explants were enhanced by moderate concentration of exogenous hormones (IAA + BA, 1.8 + 2.5 ppm) especially for root initiation and branching (6 +). Comparable results were recorded by *A. oryzae* extract cultivated on $BG11_0$ which contained 0.5 + 10 ppm. Higher IAA + BA mixture concentration (2.5 + 50 ppm) showed lesser stimulatory effect to all parameters. *A. oryzae* extracts (grown on $BG11_0$ and 100% sewage media) showed moderate shoot length and leaves' number and high root initiation of tomato explant (3 + and 5 +) compared to control, DMSO, and IAA concentration as well as IAA + BA concentration without any effect on branching and leaf expansion. The crude extract of *A. oryzae* with optimum concentration of BA showed moderate shoot length, leaves' number, branching, and root initiation (2 +)

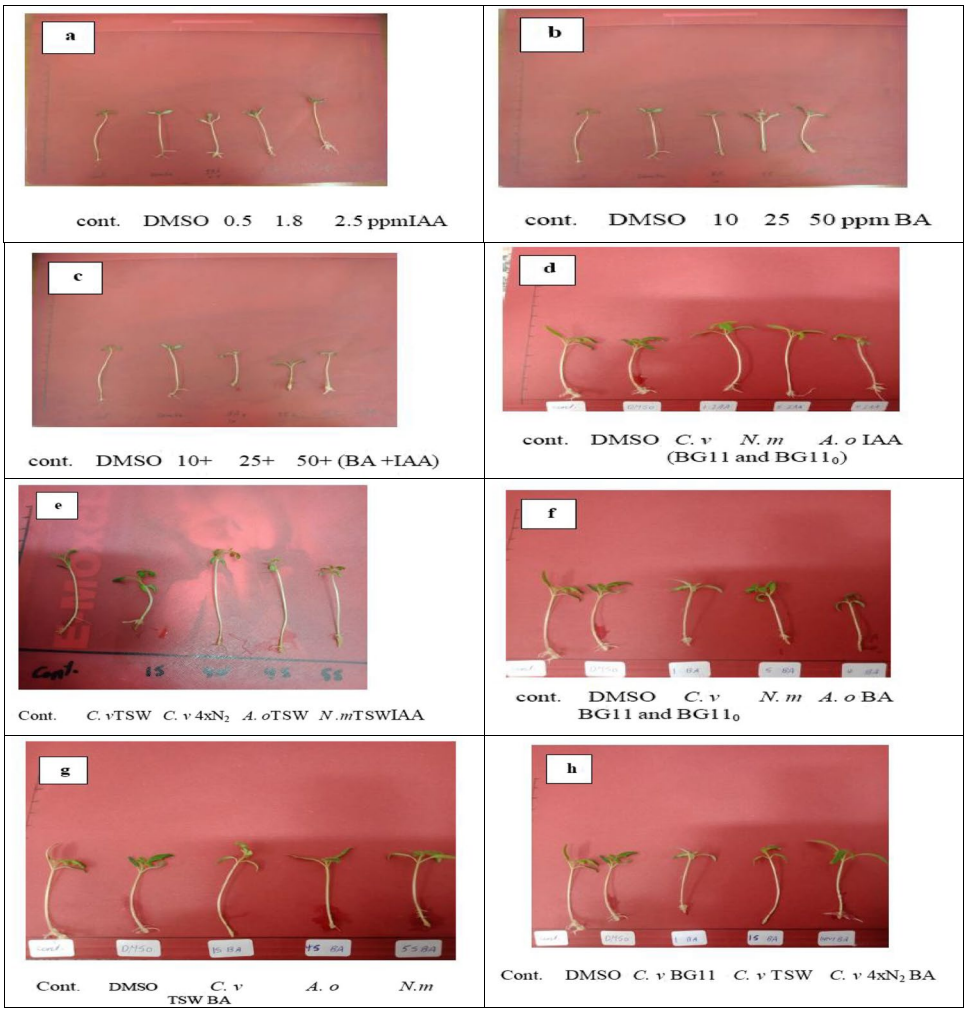

Fig. 2 Growth of tomato explants by synthetic BA and IAA and mixtures of IAA + BA concentration as well as samples and extracts (grown on various media) with optimum concentration of BA and IAA (from dose response curve) on tomato plant

Table 10 The effect of *Anabaena oryzae* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on tomato plant

Parameter	Shoot length cm	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{bcd}	3.500 ± 0.674^{a}	+ + +	-	-
DMSO	5.083 ± 0.925^{cd}	3.417 ± 0.669^{a}	+ + +	-	-
IAA (0.5 ppm)	4.850 ± 0.454^{cde}	3.083 ± 0.900^{ab}	+ + + + + +	-	-
IAA (1.8 ppm)	6.850 ± 1.588^{a}	3.583 ± 0.515^{a}	+ + + + + +	-	-
IAA (2.5 ppm)	$6.008 \pm 0.734^{a\,b}$	3.750 ± 0.452^{a}	+ + +	-	-
IAA + BA (0.5 + 10) ppm	4.008 ± 0.478^{e}	3.167 ± 0.577^{ab}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	$4.533 \pm 0.414^{d\,e}$	3.667 ± 0.492^{a}	+ + + +	+	-
IAA + BA (2.5 + 50) ppm	$4.525 \pm 0.398^{d\,e}$	2.500 ± 0.674^{b}	+	-	-
A. oryzae ($BG\text{-}11_0$)	5.208 ± 0.348^{bcd}	2.583 ± 0.515^{b}	+ + +	-	-
A. oryzae (100% TSW)	5.708 ± 1.053^{bc}	3.750 ± 0.452^{a}	+ + + + +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest

DMSO, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

Table 11 The effect of *Anabaena oryzae* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on tomato plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{a}	3.500 ± 0.674^{ab}	+ + +	-	-
DMSO	5.083 ± 0.925^{b}	3.417 ± 0.669^{ab}	+ + +	-	-
BA (10 ppm)	4.533 ± 0.624^{bc}	2.833 ± 0.577^{bc}	+	-	+
BA (25 ppm)	$4.442 \pm 0.516^{b\,c\,d}$	3.500 ± 0.674^{ab}	+	+ +	+
BA (50 ppm)	$4.425 \pm 0.636^{b\,c\,d}$	$3.333 \pm 0.651^{a\,b}$	-	+ + +	+ + +
IAA + BA (0.5 + 10) ppm	4.008 ± 0.478^{d}	3.167 ± 0.577^{abc}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	$4.533 \pm 0.414^{b\,c\,d}$	3.667 ± 0.492^{a}	+ + + +	+	-
IAA + BA (2.5 + 50) ppm	$4.525 \pm 0.398^{c\,d}$	2.500 ± 0.674^{c}	+	-	-
A. oryzae ($BG11_0$)	4.8833 ± 0.3433^{b}	2.500 ± 0.522^{c}	+ +	-	-
A. oryzae (100%TSW)	4.767 ± 0.543^{b}	3.083 ± 0.669^{abc}	+ + +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

Table 12 The effect of *Nostoc muscorum* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on tomato plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{bcd}	3.500 ± 0.674^{a}	+ + +	-	-
DMSO	5.083 ± 0.925^{cd}	3.417 ± 0.669^{a}	+ + +	-	-
IAA (0.5 ppm)	4.850 ± 0.454^{cde}	3.083 ± 0.900^{ab}	+ + + + + +	-	-
IAA (1.8 ppm)	6.850 ± 1.588^{a}	3.583 ± 0.515^{a}	+ + + + + +	-	-
IAA (2.5 ppm)	$6.008 \pm 0.734^{a\,b}$	3.750 ± 0.452^{a}	+ + +	-	-
IAA + BA (0.5 + 10) ppm	4.008 ± 0.478^{e}	3.167 ± 0.577^{ab}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	4.525 ± 0.414^{de}	3.667 ± 0.492^{a}	+ + + +	-	-
IAA + BA (2.5 + 50) ppm	$4.525 \pm 0.398^{d\,e}$	2.500 ± 0.674^{b}	+	-	-
N. muscorum ($BG\text{-}11_0$)	5.367 ± 0.350^{bcd}	3.333 ± 0.651^{a}	+ + +	-	-
N. muscorum (100%TSW)	5.692 ± 0.392^{bc}	3.750 ± 0.452^{a}	+ + + + + +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest

DMSO, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

as shown in Table 11 especially by increasing BA concentration (separately added or in moderate combination with IAA (1.8 + 25 ppm).

The obtained results of *N. muscurum* were shown in Table 12 with the tested *N. muscorum* optimum concentration on $BG11_0$ and 100% TSW media revealed that IAA (1.8 ppm) recorded the greatest shoot length (6.850 ± 1.588), leaves' number (3.583 ± 0.515), and root initiation (6 +) compared to MS and DMSO media and other IAA concentrations (0.5, 2.5 ppm) as well as the combination IAA + BA concentration, while no branching and no leaf expansion were recorded. *N. muscurum* extract (grown on $BG11_0$ and 100% TSW media) showed moderate shoot length and high leaves' number of tomato explant compared to control, DMSO, and IAA concentration as well as IAA + BA concentration which induced moderate root initiation (3 +), without branching and leaf expansion. The crude extract of *N. muscurum* with optimum concentration of BA(on $BG11_0$ and 100% TSW) had moderate shoot length (4.892 ± 0.470, 4.833 ± 0.597) and low to moderate root initiation and leaves' number (3.250 ± 0.754, 3.083 ± 0.669), as shown in Table 13.

Table 14 of the obtained results of *C. vulgaris* showed that IAA at concentration 1.8 ppm and 4 × nitrogen concentration recorded the greatest shoot length (6.850 ± 1.588, 6.817 ± 0.746) compared to those produced on MS

Table 13 The effect of *Nostoc muscorum* crude extracts (grown on $BG11_0$ and 100% TSW) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on tomato plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{a}	3.500 ± 0.674^{ab}	+ + +	-	-
DMSO	5.083 ± 0.925^{b}	$3.417 \pm 0.669^{a\,b}$	+ + +	-	-
BA (10 ppm)	4.533 ± 0.624^{bc}	2.833 ± 0.577^{bc}	+	-	-
BA (25 ppm)	4.442 ± 0.516^{bc}	3.500 ± 0.674^{ab}	+	+ +	+
BA (50 ppm)	4.425 ± 0.636^{bc}	3.333 ± 0.651^{ab}	-	+ + +	+ + +
IAA + BA (0.5 + 10) ppm	3.642 ± 0.458^{c}	3.167 ± 0.577^{abc}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	4.350 ± 0.417^{bc}	3.667 ± 0.492^{a}	+ + + +	+	-
IAA + BA (2.5 + 50) ppm	3.683 ± 0.595^{c}	2.500 ± 0.674^{c}	+	-	-
N. muscorum ($BG\text{-}11_0$)	4.892 ± 0.470^{b}	3.250 ± 0.754^{abc}	+ + +	-	-
N. muscorum (100%TSW)	4.833 ± 0.597^{b}	3.083 ± 0.669^{abc}	+ +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest; *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

Table 14 The effect of *Chlorella vulgaris* crude extracts (grown on BG11, 100% TSW, and 4 × N media) with optimum concentration of indole acetic acid (IAA) (from dose–response curve) on tomato plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{bcd}	3.500 ± 0.674^{ab}	+ + +	-	-
DMSO	$5.083 \pm 0.925^{c\,d}$	3.417 ± 0.669^{ab}	+ + +	-	-
IAA (0.5 ppm)	4.850 ± 0.454^{cde}	3.083 ± 0.900^{bc}	+ + + + + +	-	-
IAA (1.8 ppm)	6.850 ± 1.588^{a}	3.583 ± 0.515^{ab}	+ + + + + +	-	-
IAA (2.5 ppm)	6.008 ± 0.734^{ab}	$3.750 \pm 0.452^{a\,b}$	+ + +	-	-
IAA + BA (0.5 + 10) ppm	4.008 ± 0.478^{e}	3.167 ± 0.577^{abc}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	4.533 ± 0.414^{de}	3.667 ± 0.492^{ab}	+ + +	+	-
IAA + BA (2.5 + 50) ppm	4.525 ± 0.398^{de}	2.500 ± 0.674^{c}	+	-	-
C. vulgaris (BG-11)	$5.650 \pm 0.678^{b\,c}$	2.917 ± 0.996^{bc}	+ + + + + +	-	-
C. vulgaris (100%TSW)	5.050 ± 0.585 cd	$3.750 \pm 0.965^{a\,b}$	+ + + + +	-	-
C. vulgaris (4 × N)	6.817 ± 0.746^{a}	3.833 ± 0.389^{a}	+ + + + +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest; *4x N*, BG11 with fourfold of $NaNO_3$ concentration. *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

and DMSO media and other IAA concentrations (0.5, 2.5 ppm) as well as the combination IAA + BA concentration. The shoot had high leaves' number (3.583 ± 0.515, 3.833 ± 0.389) and the greatest root initiation at 1.8, 2.5 ppm IAA, and 100% TSW and 4 × N, while no branching and no leaf expansion were recorded. Both lower IAA concentration (0.5 ppm) and higher concentration (2.5 ppm) were less effective concerning shoot length and leaves' number. Morphological parameters of tomato explants were enhanced by moderate concentration of exogenous hormones (IAA + BA, 1.8 + 2.5 ppm). Comparable results were recorded by *C. vulgaris* extract cultivated on BG11 (contained 0.5 + 10 ppm). The crude extract of *C. vulgaris* with optimum concentration of BA, 100% TSW, and 4 × nitrogen concentration had moderate shoot length, leaves' number, and root initiation, without branching and leaf expansion, as shown in Table 15.

3.4 Molecular analyses

The potentiality of ISSR analyses was used to evaluate the genetic variation of canola and tomato in vitro plantlets (treated and control) (Tables 16, 17, 18, and 19) (Figs. 3 and 4).

Table 15 The effect of *Chlorella vulgaris* crude extracts (grown on BG11, 100% TSW, and 4×N media) with optimum concentration of benzyl adenine (BA) (from dose–response curve) on tomato plant

Parameter	Shoot length (cm)	Leaves' numbers	Root initiation	Branching	Leaf expansion
Control (MS medium)	5.217 ± 0.469^{a}	$3.500 \pm 0.674^{a\,b\,c}$	+ + +	-	-
DMSO	$5.083 \pm 0.925^{b\,c\,d}$	$3.417 \pm 0.669^{a\,b\,c}$	+ + +	-	-
BA (10 ppm)	$4.533 \pm 0.624^{c\,de}$	$2.833 \pm 0.577^{c\,d}$	+	-	+
BA(25 ppm)	$4.442 \pm 0.516^{c\,d\,e}$	$3.500 \pm 0.674^{a\,b\,c}$	+	+ +	+
BA (50 ppm)	4.425 ± 0.636^{de}	$3.333 \pm 0.651^{a\,b\,c}$	-	+ + +	+ + +
IAA + BA (0.5 + 10) ppm	3.642 ± 0.458^{e}	3.167 ± 0.577^{abcd}	+ + +	-	-
IAA + BA (1.8 + 25) ppm	4.350 ± 0.417^{de}	$3.667 \pm 0.492^{a\,b}$	+ + + +	+	-
IAA + BA (2.5 + 50) ppm	3.683 ± 0.595^{e}	2.500 ± 0.674^{d}	+	-	-
C. vulgaris (BG-11)	$4.808 \pm 0.543^{b\,cd}$	3.000 ± 0.603^{bcd}	+ + + +	-	-
C. vulgaris (100%TSW)	5.5750 ± 0.3306^{b}	$2.917 \pm 0.515^{c\,d}$	+ +	-	-
C. vulgaris (4×N)	$5.325 \pm 0.694^{b\,c}$	3.750 ± 0.452^{a}	+ + + +	-	-

TSW, treated wastewater; (-), absence; (6 +), the highest; (5 +), high; (4 +), moderate; (3 +), low; (+), the lowest; *4x N*, BG11 with fourfold of $NaNO_3$ concentration. *DMSO*, dimethyl sulfoxide; *MS*, Murashige and Skoog medium

Table 16 ISSR analyses of canola in vitro plantlets (control and treated) using 4 primers

Primers	Total No. of bands	Monomorphic bands	Polymorphic bands	% polymorphism
UBC811	31	20	11	35.48
UBC818	52	40	12	20.07
UBC834	67	60	7	10.45
UBC849	79	50	29	36.71
Total	229	170	59	25.76

Table 17 RAPD analyses of canola in vitro plantlets (control and treated) using 4 primers

Primers	Total No. of bands	Monomorphic bands	Polymorphic bands	% polymorphism
B12	72	60	12	16.67
P13	92	72	20	21.74
N8	89	50	39	43.82
C1	69	50	19	27.54
Total	322	232	54	16.77

Table 18 ISSR analyses of tomato in vitro plantlets (control and treated) using 4 primers

Primers	Total No. of bands	Monomorphic bands	Polymorphic bands	% polymorphism
UBC811	40	36	4	10.00
UBC818	50	0	50	100
UBC834	47	20	27	57.45
UBC849	43	10	33	76.74
Total	180	66	114	63.33

Table 19 RAPD analyses of tomato in vitro plantlets (control and treated) using 4 primers

Primers	Total No. of bands	Monomorphic bands	Polymorphic bands	% polymorphism
B12	80	0	80	100
P13	89	30	59	66.29
N8	54	0	54	100
C1	38	20	18	47.37
Total	261	50	211	80.84

Table 16 revealed that a total of 229 bands were generated from canola in vitro plantlets using 4 ISSR primers with an average of 25.76 bands per primer. Each primer produced a unique banding pattern of 7 (using UBC834), 11 (using UBC811), 12 (using UBC818), and 29 (using UBC849) amplicons. UBC849 primer exhibited the highest discrimination between the plantlets showing the formation of 29 polymorphic bands (36.71%). On the other hand, UBC834 had the lowest differentiation potential as it revealed 7 polymorphic bands (10.45%) between the plantlets (Table 16). Figure 3a revealed that the 4 ISSR primers indicated that canola plantlets grown on *A. oryzae* extract [contain either BA (5C) or IAA (6C)] or on *C. vulgaris* extract (4×N) [contain IAA (9C) or BA (10C)] had low divergence compared to controls. Concerning RAPD, a total of 322 bands were produced using 4 primers with an average of 16.77 bands

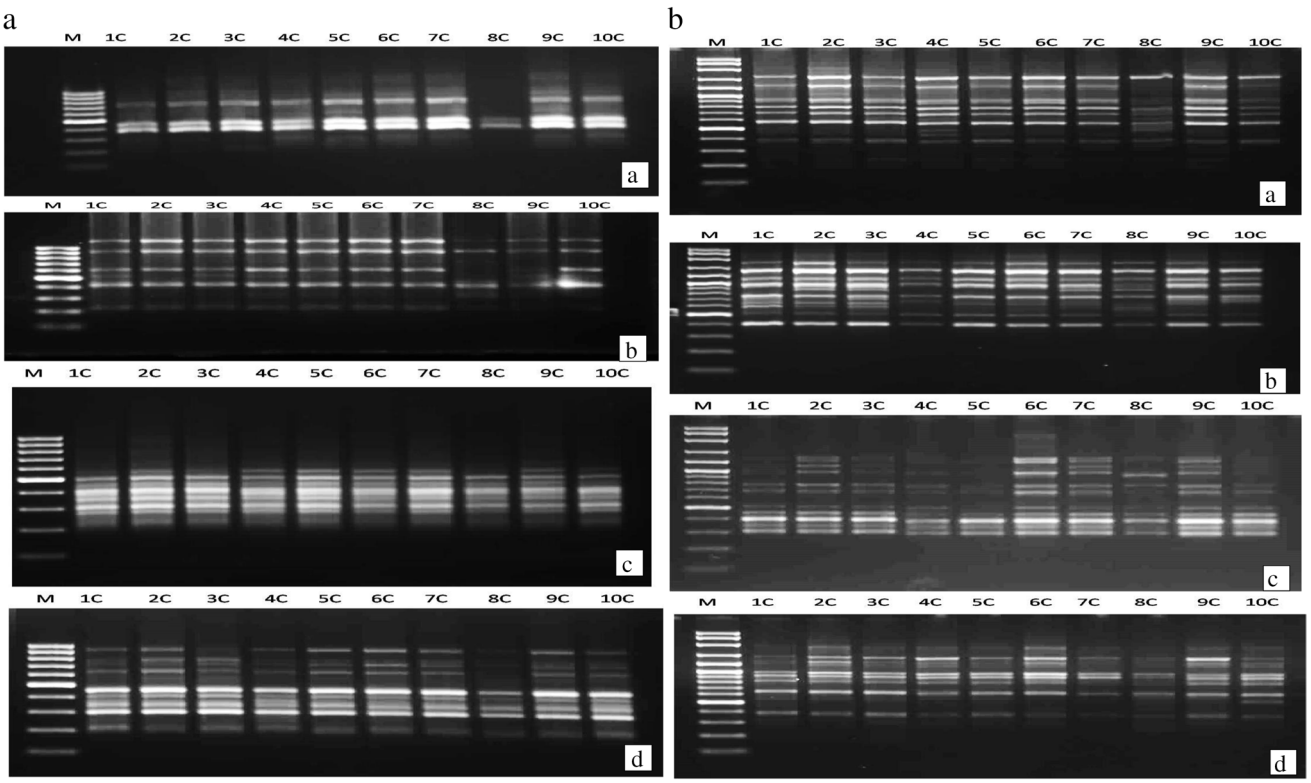

Fig. 3 **a** ISSR banding patterns of canola in vitro plantlets (control and treated) using 4 primers: (a) UBC811; (b) UBC818; (c) UBC834, and (d) UBC849 marker (M) 3000 bp. 1C, control (MS); 2C, control (DMSO) and treatments (3C-10C); 3C, 1.8 ppm IAA + 25 ppm BAA; 4C, 1.8 ppm IAA; 5C, *A.oryzae* ext. contained BA (grown on 100% TSW); 6C, *A. o*ryzae ext. contained IAA (grown on 100% TSW); 7C, *N. muscurum* ext. contained IAA (grown on 100% TSW); 8C, *N. muscurum* ext. contained BA (grown on 100% TSW); 9C, *C. vulgaris* ext. contained IAA (grown on 100% TSW); 10C, *C. vulgaris* ext. contained BA (grown on 100% TSW). **b** RAPD banding patterns of canola in vitro plantlets (control and treated) using 4 primers: (a) B12; (b) P13; (c) N8, and (d) C1. Marker (M) 3000 bp; 1C, control (MS); 2C, control (DMSO) and treatments (3C-10C); 3C, 1.8 ppm IAA + 25 ppm BAA; 4C, 1.8 ppm IAA; 5C, *A.oryzae* ext. contained BA (grown on 100% TSW); 6C, *A. o*ryzae ext. contained IAA (grown on 100% TSW); 7C, *N. muscurum* ext. contained IAA (grown on 100% TSW); 8C, *N. muscurum* ext. contained BA (grown on 100% TSW); 9C, *C. vulgaris* ext. contained IAA (grown on 100% TSW); 10C, *C. vulgaris* ext. contained BA (grown on 100% TSW)

per primer (Table 17). The primers produced a banding pattern of 69 (using C1), 72 (using B12), 89 (using N8), and 92 (using P13) amplicons. N8 primer exhibited the highest differentiation between the plantlets showing the formation of 39 polymorphic bands (43.82%). B12 had the lowest discrimination ability as it revealed 12 polymorphic bands (16.67%) between the canola plantlets. Figure 3b disclosed that canola plantlets grown on *A.oryzae* extract (C5 and C6) were nearly similar to the controls using 3 RAPD primers (B12, P13, and C1), but N8 primer clarifies the difference between them and the controls. The figure also revealed that the plantlets grown on other algal species extracts expressed high genetic divergent compared to others.

The prospect of ISSR and RAPD analyses was applied to assess the genetic divergence between tomato in vitro plantlets (treated and control) (Tables 18 and 19) (Fig. 4a, b). Regarding ISSR, a total of 180 bands were created using 4 primers with 63.33% polymorphism. The used primers produced special banding pattern ranging from 40 to 50 amplicons. UBC818 primer exhibited complete discrimination between the plantlets showing the formation of 100% polymorphism. On the other hand, UBC811 had the lowest differentiation potential as it revealed only 4 polymorphic bands (10%) between the plantlets. Figure 4a revealed that the 3 ISSR primers (UBC 811, UBC 834, and UBC849) indicated that the plantlets grown on *A.oryzae* extract [contain either BA (5 T) or IAA (6 T)] or on *N. muscurum* extract [contain either BA (7 T) or IAA (8 T)] had low divergence compared to controls, but the primer (UBC 818) revealed the differences between each other and controls. Figure 4a also revealed that the plantlets grown on mixture of synthetic hormones (3 T) have high genetic variations compared to others. In case of RAPD, a total of 261 bands were produced using 4 primers with an average of 80.84% polymorphism (Table 19 and 20). The 4 primers produced banding pattern of 38 (using C1), 54 (using N8), 80 (using B12), and 89 (using P13) amplicons. B12 primer showed the highest variations between the plantlets producing 100% polymorphism.

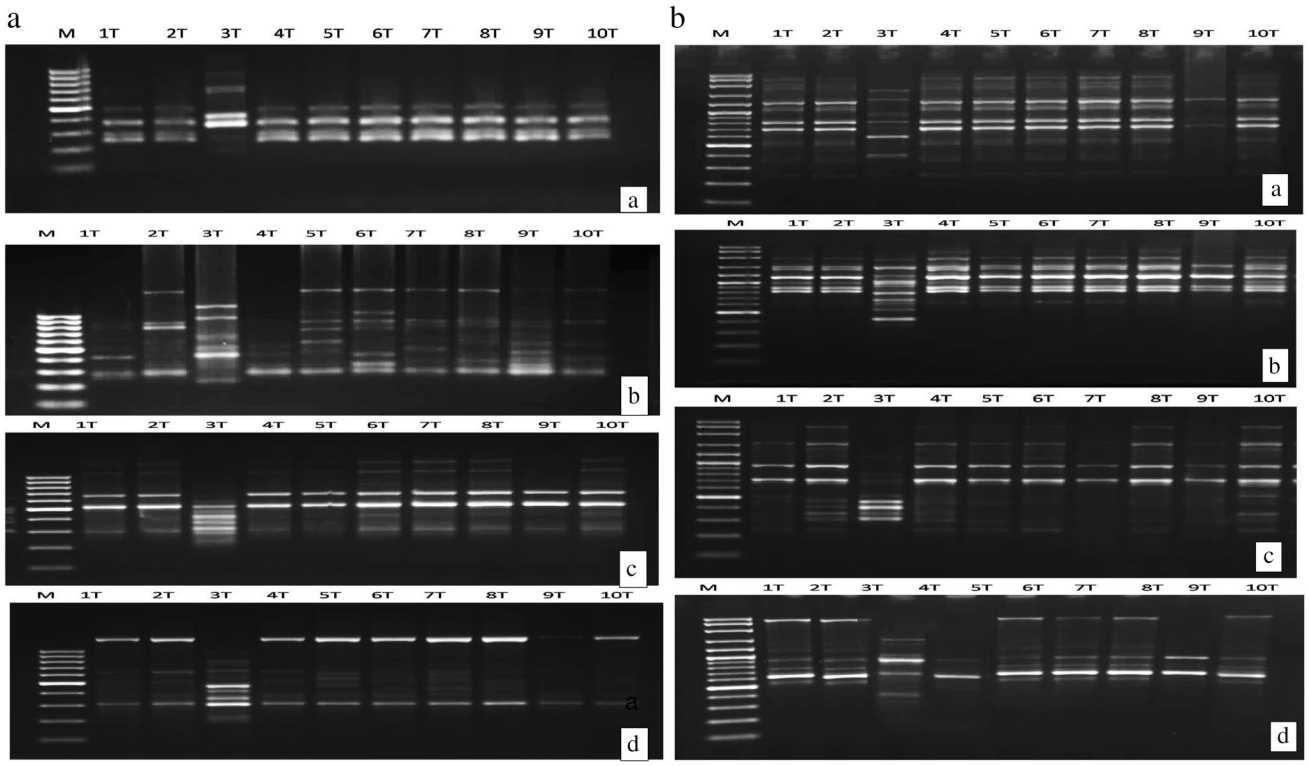

Fig. 4 **a** ISSR banding patterns of tomato in vitro plantlets (control and treated) using 4 primers: (a) UBC811; (b) UBC818; (c) UBC834, and (d) UBC849. Marker (M) 3000 bp. 1 T, control (MS); 2 T, control (DMSO) and treatments (3 T-10 T); 3 T, 1.8 ppm IAA + 25 ppm BA; 4 T, 1.8 ppm IAA; 5 T, *A.oryzae* ext. contained BA (grown on 100% TSW); 6 T, *A. o*ryzae ext. contained IAA (grown on 100% TSW); 7 T, *N. muscurum* ext. contained IAA (grown on 100% TSW); 8 T, *N. muscurum* ext. contained BA (grown on 100% TSW); 9 T, *C. vulgaris* ext. contained IAA (grown on 4×N); 10 T, *C. vulgaris* ext. contained BA (grown on 4×N). **b** RAPD banding patterns of tomato in vitro plantlets (control and treated) using 4 primers: (a) B12; (b) P13; (c) N8, and (d) C1; marker (M) 3000 bp; 1 T, control (MS); 2 T, control (DMSO) and treatments (3 T-10 T); 3 T, 1.8 ppm IAA + 25 ppm BA; 4 T, 1.8 ppm IAA; 5 T, *A.oryzae* ext. contained BA (grown on 100% TSW); 6 T, *A. o*ryzae ext. contained IAA (grown on 100% TSW); 7 T, *N. muscurum* ext. contained IAA (grown on 100% TSW); 8 T, *N. muscurum* ext. contained BA (grown on 100% TSW); 9 T, *C. vulgaris* ext. contained IAA (grown on 4×N); 10 T, *C. vulgaris* ext. contained BA (grown on 4×N)

Table 20 ISSR and RAPD Primer sequences

Marker	Primer name	Sequences
ISSR	UBC811	GAG AGA GAG AGA GAG AC
	UBC818	CAC ACA CAC ACA CAC AG
	UBC834	GAGAGAGAGAGAGAGAGAGAT
	UBC849	GAGAGAGAGAGAGAGAT
RAPD	B12	CCTTGACGCA
	P13	GGAGTGCCTC
	N8	ACCTCAGCTC
	C1	TTCGAGCCAG

C1 had the lowest discrimination potential as it revealed 18 polymorphic bands which represented 47.37% polymorphism between the tomato plantlets. Figure 4b disclosed that the plantlets grown on *A. oryzae* extract [contain either BA (5 T) or IAA (6 T)] and on *N. muscurum* extract [contain either BA (7 T) or IAA (8 T)] had less polymorphism and were nearly similar to the controls. Figure 4b also revealed that the plantlets grown on mixture of synthetic hormones (3 T, 1.8 ppm IAA + 25 ppm BAA) have high genetic variations compared to others.

From all the above data, we can reveal that the whole banding pattern of in vitro plantlets of both species using 4 ISSR and 4 RAPD primers that canola plantlets grown on *A. oryzae* extract or on *C. vulgaris* extract (4×N) had low divergence compared to controls, while the tomato plantlets grown on *A.oryzae* extract and on *N. muscurum* extract had less polymorphism and were nearly similar to the controls. However, the tomato plantlets grown on mixture of synthetic hormones have high genetic variations compared to others.

Plant biotechnology is essential to regenerate elite in vitro plantlets through micropropagation. So, it becomes important to use molecular markers for screening genetic variations among the raised plantlets in order to select true to type progenies. Inter-simple sequence repeat (ISSR) technique is a powerful PCR-based technique and broadly used for

detecting genetic variations among in vitro raised plantlets [31–33]. This method is simple, discriminative, cost-effective, and authentic. It uses microsatellites as primers, usually 16–25 bp long, for selecting these multiple genomic loci without the need of previous knowledge on sequences of the target organism genome [34, 35].

Plant biotechnology is essential to regenerate elite in vitro plantlets through micropropagation. So, it becomes important to use molecular markers for screening genetic variations among the raised plantlets in order to select true to type progenies. ISSR technique is a powerful PCR-based technique and broadly used for detecting genetic variations among in vitro raised plantlets [31–33]. This method is simple, discriminative, cost-effective, and authentic. It uses microsatellites as primers, usually 16–25 bp long, for selecting these multiple genomic loci without the need of previous knowledge on sequences of the target organism genome [34, 35].

No available literature studied intraspecific genetic variation of in vitro plantlets treated with seaweed hormonal extract using ISSR markers.

Compared with our results, Al-Zubaidy and Al-Hamzawi [36] revealed the formation of genetic variants between control and sweet pepper plantlets treated with 6 mg/L seaweed extract, recording percentage polymorphism from 16.67 to 33.33%, employing 5 RAPD markers.

In contrary with our results, Yadav et al. [37] revealed that the amplification products of the regenerated plants derived from tuber explants of Malabar glory lily (*Gloriosa superba* L.) cultured on MS media supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA had similar banding patterns to that of the mother plant using 10 RAPD and 7 ISSR primers. Similarly, Goyal et al. [38] found that all in vitro-regenerated plantlets (*Dendrocalamus strictus*) derived from buds of nodal cuttings and cultured on MS media supplemented with synthetic hormones showed clonal fidelity when assessed by RAPD and ISSR markers. Also, Faisal et al. [32] reported that all in vitro plantlets of *Ruta graveolens* grown on MS media with synthetic hormones expressed genetic fidelity to the mother plant using 20 RAPD and ISSR markers. In addition, Borsai et al. [39] reported that all micropropagated plantlets of blackberry cultured on MS medium supplemented with 0.5 mg/l BA had no polymorphism when analyzed them by RAPD and SRAP markers.

4 Conclusion

From the obtained results, we can conclude that the use of treated wastewater as nutritive media for cultivation of microalgae is suitable and not expensive methods when compared with the ordinary synthetic cultivation nutrient methods. In addition, algal strains under this stress condition enhanced their ability for the production of plant growth regulators (PGRs) and other active ingredients to which attributed to be considered as good safe bio-fertilizers which produces low genetic variations compared to the synthetic PGRs.

Data availability The data used and analyzed in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

1. Wallmann F, Dietze S, Ackerman J-U, Walther T, Steingroewer J, Krujatz F (2019) Microalgae wastewater treatment. Engineering in Life Sciences 19(12):860–871
2. Al-Jabri H, Das P, Khan S, Thaher M, AbdulQuadir M (2021) Treatment of wastewaters by microalgae and the potential application of the produced biomass–A review. Water 13(1):27. https://doi.org/10.3390/w13010027
3. Mohsenpour SF, Hennige S, Willoughby N, Adeloye A, Gutierrez T (2020) Integrating micro-algae into wastewater treatment: a review. Sci Total Environ 752(15):142168
4. Wang L, Min M, Li Y, Chen P, Chen Y, Liu Y, Wang Y, Ruan R (2010) Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 162(4):1174–1186
5. Hashem HA, Masour HA, El-Khawas SA, Hassanein RA (2019) The potentiality of marine macro-algae as bio-fertilizer to improve the productivity and salt stress tolerance of canola (*Brassica napus* L.) plants. Agronomy 9(146):1–17
6. Hussain HI, Kasinadhuni N, Arioli T (2021) The effect of seaweed extract on tomato plant growth, productivity and soil. J Appl Phycol. https://doi.org/10.1007/s10811-021-02387-2
7. Khan W, Rayirat VP, Subramanian S, Jithesh MN, Rayorath P, Hodges DM, Critchley AT, Craigie JS, Norrie J, Balakrisham P (2009) Seaweed extracts as biostimulants of plant growth and development. J Plant Growth Regul 28:386–399
8. Puglisi I, La Bella E, Rovetto EI, Lo Piero AR, Baglier A (2020) Biostimulant effect and biochemical response in lettuce seedlings treated with *Scenedesmus quadricauda* extract. Plants 9(123):1–13
9. Naz S, Siddiqui MF, Raza S (2018) Effect of different growth regulators on in vitro propagation of *Brassica napus L.* Pak J Bot 50(5):1871–1876
10. Vanil R, Haq AU, Agnihotri RK (2010) Plant growth regulators as effective tool for germination and seedling growth for *Bambusa arundinacea*. Res J Agric Sci 1:233–236
11. Zheng S, Jiang Jie, He M, Zou S, Wang C (2016) Effect of kelp waste extracts on the growth and development of Pakchoi (*Brassica chinensis L.*). Sci Rep 6:38683
12. Shariatmadari Z, Riah H, Hashtroudi MS, And GAR, Aghashariatmadary Z (2013) Plant growth promoting cyanobacteria and

their distribution in terrestrial habitats of Iran. Siol Sci Plant Nutr 59:535–547
13. Kumar M, Agnihotri RK, Vanil R, Sharma R (2014) Effect of phytohormones on seed germination and seedling growth on *Coriandrum sativum* L. Paki J Biol Sci 17(4):594–596
14. Suresh A, Soundararajan S, Elavarasi S, Lewis OF, Thajuddin N (2019) Evaluation and characterization of the plant growth promoting potentials of two heterocystous cyanobacteria for improving food grains growth. Biocatal Agric Biotechnol 17:647–652
15. Rani V, Raina SN (2020) Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol–Plant 36:319–330
16. Goncalves AL (2021) The use of microalgae and cyanobacteria in the improvement of agricultural practices: a review on their biofertilising, biostimulating and biopesticide roles. Appl Sci 11:871. https://doi.org/10.3390/app11020871
17. Nekvapil F, Ganea I-V, Ciorîță A, Hirian R, Tomšić S, Martonos IM, Cintă Pinzaru S (2021) A new biofertilizer formulation with enriched nutrients content from wasted algal biomass extracts incorporated in biogenic powders. Sustainability 13:3777. https://doi.org/10.3390/su13168777
18. Umamaheswari J, Shanthakumar S (2020) Paddy-soaked rice mill wastewater treatment by phycoremediation and feasibility study on use of algal biomass as biofertilizer. Wiley on line liberary. com. https://doi.org/10.1002/jctb.6551.
19. Heinz B, Schmidt J (1995) Monitoring genetic fidelity vs somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. Euphytica 85:341–345
20. Rani V, Raina SN (1996) PCR technology. Botanica 46:78–81
21. Largia MJV, Pandian SK, Ramesh M (2013) Genetic fidelity assessment of encapsulated in vitro tissues of Bacopa monnieri after 6 months of storage by using ISSR and RAPD markers. Turk J Bot 37:1008–1017
22. Rippka R, Deruelles J, John J, Waterbury B, Herdman M, Roger Y (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61
23. Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol Rev 35:171–205
24. APHA (1998) Standard methods for the examination of water and wastewater, 19th edn. American Public Health Association, Washington DC (**ISBN: 0-8-553060-5**)
25. Elakbawy WM, Shanab SMM, Shalaby EA (2021) Biological activities and plant growth regulators producing from some microalgae biomass cultivated in different wastewater concentrations. Biomass Conver Biorefin. https://doi.org/10.1007/s13399-021-01610-x
26. Sun FuJ, Wang X, Chu J, Yan C (2011) Progress in quantitative analysis of plant hormones. Chin Sci Bull 56:355–366
27. Gamborg OL, Phillips GC (eds) (1995) Plant cell, tissue and organ culture: fundamental methods. Springer-Verlag, Berlin Heidelberg, pp 45–54
28. Murashige T, Sookg F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physio Plant 15:473–497
29. Doyle JJ, Doyle JL (1990) Isolation of DNA from fresh tissue. Focus 12:13–15
30. Snedecor G, Cochran W (1982) Statistical methods. Iowa state University Press, Iowa, p 511
31. Ahmad N, Anis M (2011) An efficient in vitro process for recurrent production of cloned plants of Vitex negundo L. Eur J For 130:135–144
32. Faisal M, Ahmad N, Alatar AA, Qahtan AA (2018) Auxin-cytokinin synergism in vitro for producing genetically stable plants of *Ruta graveolens* using shoot tip meristems. Saudi J Biol Sci 25:273–277
33. Joshi P, Dhawan V (2007) Assessment of genetic fidelity of micropropagated Swertia chirayita plantlets by ISSR marker assay. Biol Plant 51:22–26
34. Muzila M, Werlemark G, Ortiz R, Sehic J, Fatih M, Setshogo M, Mpoloka W, Nybom H (2014) Assessment of diversity in *Harpagophytum* with RAPD and ISSR markers provides evidence of introgression. Hereditas 151(4–5):91–101
35. Vanijajiva O (2012) The application of ISSR markers in genetic variance detection among Durian (*Durio zibethinus* Murr.) cultivars in the Nonthaburi province, Thailand. Procedia Eng 32:155–159
36. Al-Zubaidy IA, Al-Hamzawi MA (2020) DNA molecular characterization of Sweet Pepper cv. Flavio as affected by seaweed extract and amino acids. IOP Conf Ser Earth Environ Sci 553:012040. https://doi.org/10.1088/1755-1315/553/1/012040
37. Yadav K, Aggarwal A, Singh N (2013) Evaluation of genetic fidelity among micropropagated plants of *Gloriosa superba* L. using DNA-based markers - a potential medicinal plant. Fitoterapia 89:265–270
38. Goyal AK, Pradhan S, Basistha BC, Sen A (2015) Micropropagation and assessment of genetic fidelity of *Dendrocalamus strictus* (Roxb.) nees using RAPD and ISSR markers. 3 Biotech 5(4):473–482
39. Borsai O, Hârța M, Szabo K, Kelemen C, Andrecan FA, Codrea M, Clapa D (2020) Evaluation of genetic fidelity of in vitro-propagated blackberry plants using RAPD and SRAP molecular markers. Hort Sci (Prague) 47:21–27