Biometric and Genetic Stability Assessment of In Vitro Rooted Populus alba L. Micro-Shoots

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Title page

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Abstract

Populus alba is a deciduous large woody tree. The plant was introduced to shooting, then rooting multiplication on MS media. This work's design depends on two factors (media power and sucrose concentrations) with different concentrations resulted in 12 treatments. There were nine responses measured in the new individuals. Some morphological parameters had a significant difference, such as shoot length, leaf and root numbers. Where in the physiological measurements, all the parameters measured had significant differences. RAPD-PCR molecular marker was applied to assess the variation in the new individuals' genetic stability from treatments with the two factors. Four RAPD primers gave reproducible bands and resulted in a total polymorphism percentage of 38.33%. The new individuals resulted from the tissue culture technique were identical to the mother plant and each other. However, in this work, the use of different media power and sucrose concentrations resulted in different values in morphological, physiological responses, and *P. alba's* molecular variation.

Keyword: Chlorophyll; Genetic Stability; *Populus alba*; RAPD-PCR; Soma clonal variations.

1. Introduction

The genus *Populus* is widely distributed in the whole world, especially in the Northern hemisphere. It belongs to the family Salicaceae [1, 2]. *Populus* is the main model system for genomic, genetic and physiological research on trees [3]. It is an essential model for woody perennial biotechnology because it is amenable to *in vitro* culture and genetic engineering through *Agrobacterium*-mediated transformation [4]. It was the first tree in which the genome has been sequenced [5]. *Populus alba* (White poplar) is a native to the Mediterranean region. It is a fast-growing, deciduous tree. White poplar leaves are used as bio-monitors of soil pollution [6]. Poplar trees propagated through sucker shoots that arise from horizontal roots and rapid growth [7]. Aspen (*Populus tremula* and *P. tremuloides*) are, however, difficult to root from woody cuttings [8]. Thus, an efficient *in vitro* propagation system for aspen is highly demanded.

Populus alba was one of the first objectives of in vitro propagation trials. The previous studies founded the vegetative propagation established from a single bud and different originated callus-based plant regeneration. In the beginning, there were difficulties of culture establishment and genetically determined differences between the species.

The success of the establishment depends on the age of the mother plants. Recently, the development of micropropagation methods of poplars is for commercial purposes with media optimization. Breeding work based on *in vitro* explants started almost parallel with developing an *in vitro* mass-propagation procedure for poplars, based on protoplast and cell suspension production followed by plant regeneration [9].

In vitro, culture studies integrated some molecular markers to examine variable changes in resulted plantlets. There are many molecular markers, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and inter simple sequence repeats (ISSR). They were used to estimate genomic polymorphism among different transformed plant lines and its non-transgenic ones [10, 11]. Also, used for detecting polymorphism at the DNA level. For example, RAPD-PCR gained much popularity because it is simple and doesn't require prior information on nucleotide sequence. RAPD-PCR can be performed with a minimal amount of genomic DNA. RAPD technique is simple, efficient, reliable, and an economical means of cultivar identification and diversity analysis [12].

In various studies, some plants' genetic diversity has been investigated using different molecular markers [13]. The most effective uses have been the study of molecular variability and phylogenetic relationships, marker-assisted selection, varietal identification, quantitative trait loci (QTLs), or the map-based cloning of genes [14]. Despite using different molecular markers to examine genetic diversity in cultivated plant species, many identify a limited level of polymorphism. Thus, identifying more polymorphic molecular markers is essential for research [15].

Phenotypic, physiological and genetic variations were reported due to the propagation process with different media compositions. Therefore, it is essential to assess the genetic stability of *Populus alba*. This study monitors the genetic stability of long-term micro-propagated shoots of *P. alba* using the RAPD-PCR molecular technique. None of these studies were previously investigated for the *in vitro* propagation of *P. alba*.

2. Materials and methods

2.1. Plant materials

Nodal segments explants of *Populus alba* were brought from Horticulture Research Station, Al-Qalyubia Governorate. Then they were brought to the tissue culture lab in of ACGEB (Agriculture Center for Genetic Engineering and Biotechnology (ACGEB), in Faculty of Agriculture, Ain Shams University). *P. alba* stem node segments were sterilized and cultured on free MS media for two months. The explants were defoliated and washed

carefully in fluid tap water to eliminate all the stacked dust/soil practices. The explants' surface sterilization was 20% Clorox + 0.1% $HgCl_2$ for 20 minutes and washed 4 to 5 times with double distilled sterile water. Excising and procedure of culture for explants stem nodal was performed under sterilized condition. MS basal medium contains required nutrients of macro-and micro-elements for the in vitro cultured plants described by [16]. The medium was allocated into incubation jars, where each jar contained 60 ml. Stem nodal cultures were incubated at 25 ± two °C and sufficient fluorescent light of 3000 Lux for 16-hour photoperiod provided by cool, white fluorescent lamps.

Resulted shoots from the establishment were excised and transferred into a multiplication medium of MS supplemented with 0.075 mg/l of BAP to obtain micro-shoots required for the rooting experiment.

2.2. Method

2.2.1. *In Vitro* Rooting Experiment

Populus alba stem node segments were sterilized and cultured on free MS media for two months. *P. alba* was maintained on MS tissue culture media according to [16]. For root formation, the shoots were developed on free MS multiplication media supplemented with 0.5 g/L activated charcoal. After that, they were transferred and cultured in 400 ml jars containing 60 ml MS media.

2.2.2. Experimental Design

Design factors are parameters that can influence a treatment's performance in general and response variables in particular. In this study, there are two main factors (media power and Auxins hormone concentration). Four nodal segments/jar (about 10–15 mm long) were cultured on ¼, ½ and ¾ MS basic medium supplemented with different sucrose concentrations at (0.0, 5, 10 and 15 g). The shoots were maintained for four weeks under the same culture conditions for developing shoots (16h light/8h dark).

2.2.3. Morphological measurements

The following seven morphological biometric parameters were measured for all these treatments: plantlet fresh weight, shoot length, root length, leaf length, leaf number, and roots.

2.2.4. Physiological measurements

The physiological parameters were measured as photosynthetic pigments, total proteins and total soluble sugars of various rooting treatments of *P. alba* plantlets.

2.2.4.1. Determination of Photosynthetic pigments

Chlorophyll a, b and carotenoids were determined in the fresh leaves of the plantlets, according to [17]. A known weight of fresh leaves was homogenized in 85% acetone. After centrifugation, the supernatant, which contained the pigments, was made up to a definite volume with 85% acetone. The extract measured against a blank of pure 85% aqueous acetone at three wavelengths of 452, 645 and 664 nm using a colorimeter. The concentration of chlorophyll a, b and carotenoids were calculated as µg/ml using the following equations:

$$Chlorophyll\ a = 10.\ 3\ E664 - 0.918\ E645 \quad (1)$$

$$Chlorophyll\ b = 19.\ 7\ E645 - 3.87\ E664 \quad (2)$$

$$Carotenoids = 4.\ 3\ E452\ (0.\ 0265\ Chl.\ a + 0.\ 426\ Chl.\ b) \quad (3)$$

Then, the fractions were calculated as mg/g fresh weight:

$$\frac{Fraction \times dilution}{1000}\ mg/g \quad (4)$$

2.2.4.2. Total Protein

The total protein was extracted from *Populus* leaves, according to [18] as follows: Weight 0.5 g of leaves were weighted and grind well with 0.5 ml of [2x] buffer. Then vortex 10 min and centrifuge for 15 min at 14000 rpm at 4°C. The supernatant contained the total protein content of plant species. Finally, the protein concentration was estimated according to [18] as follows: 0.1ml of supernatant was pipette into a test tube, and 5ml of protein reagent was added, mixed, and measured by Spectrophotometer at wavelength 595 nm. The concentration of protein was determined from the protein standard curve. The concentration was calculated according to the following equation: X (conc.) = (Y (abs.) – 0.030) / 0.007 (5).

2.2.4.3. Total Soluble Sugars (TSS)

A Known weight of leaves was ground in 5ml ethanol 70%, after centrifugation, the supernatant was completed to a known volume by distilled water. Total sugars were determined using the anthrone technique as described by [19]. Six ml anthrone solution (2g/l LH_2SO_4 95%) was added to the 3 ml sample and maintained on a boiling water-bath

for 3 min. After cooling, the developed color was measured spectrophotometrically at 620 nm. Then use equation (6) to compute the concentration of the total soluble sugars

Both total phenolic compounds and total flavonoids content were calculated, according to the following equation: –

$$\text{Concentration (mg/g)} = \frac{(R-B)*dilution\ factor*factor}{1000} \quad (6)$$

**R: reading of samples at spectrophotometer, B reading of blank at spectrophotometer*

2.2.5. Molecular marker

2.2.5.1. DNA isolation and RAPD-PCR bioassay

The total genomic DNA of *Populus alba* (different treatments) was isolated using the CTAB method according to [20]. 0.5 g of leaves were ground with 700 µl of 2% CTAB buffer and incubated at 65°C for 30 min with vortex every 10 min. The Eppendorf tubes were centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred into new tubes. An equal volume of chloroform: isoamyl alcohol (24:1) was added to each tube and set for 2 min at room temperature, then centrifuged at 12,000 rpm for 10 min at 4°C. The upper aqueous layer was transferred into new tubes and add 800 µl of absolute ice-cold ethanol and left for about two hours at -20°C. Centrifuge to precipitate DNA pellets and then wash them with 70% ice-cold ethanol. Finally, resuspend the pellets in 50 µl of TE buffer and keep them at -20°C till applying the RAPD-PCR reaction.

Seven RAPD decamer primers were used in this bioassay. Only 4 of them gave reproducible clear bands. These primers were listed in table (3). RAPD-PCR reaction was carried out in Biometra thermocycler. The reaction mixture was carried out in a total volume of 25 µl containing 12.5 µl Taq master mix (COSMO PCR RED M. Mix, W1020300x), 2 µl of genomic DNA, 1 µl for each primer (Willowfort) and 9.5 µl ddH_2O. The reaction program was 35 cycles of the following steps: Denaturation for 30 sec at 94°c, annealing 30 sec at different degrees for each primer as shown in table (4) and extension for 1 min at 72°c. This was followed by one step of final extension at 72°c for 10 min then cooling at 4°c. The amplified PCR product was run on 1.2% agarose gel compared to (New England Biolab, #N3232S) ladder.

2.2.6. Statistical Analysis

The gel electrophoresis images were analyzed, as the presence of a band was scored as 1, whereas the band's absence was coded as 0. A pairwise similarity matrix was generated using Jaccard's similarity coefficient. Using the

unweighted pair group method with the arithmetic averaging algorithm (UPGMA), cluster analysis was performed to develop a dendrogram. These computations were carried out using Bio-Rad Quantity one (4.6.2) [21].

Data collected were subjected to analysis of variance test in SPSS 21. Mean average, standard deviations, and correlations were estimated. Significant means were separated using Tukey test multivariate analysis. Tukey homogeneous Subsets were applied for the different 25 treatments using one-way ANOVA. Where Tukey homogeneous Subsets were applied for the two factors using multivariate analysis. Community Package Analysis (CPA, 1.2) software was used to assess the relation between all Populus treatment*s* based on morphological, physiological, and molecular data. CAP tools were complete linkage clustering and PCA blot of PCA covariance ordination.

3. Results and Discussion

3.1. Morphological and Physiological Parameters

Result data in Table (1) shows that media power and various sucrose concentrations had significant effects on shoot length leaf number and root number. The media power of ½ and 15g/L sucrose gave the most significant yield in shoot length. In the case of leaf number, ¼ media power with nearly all the different sucrose concentrations gave the high number. In contrast, ¼ media power with almost all the sucrose concentrations enhances rooting in *Populus*.

The different physiological measured parameters' response was estimated, and the average was recorded in table (2). The measured pigmentation (chlorophyll a, chlorophyll b, carotenoids and Total pigmentation), total protein and total soluble sugars showed significant difference. In the case of all pigments (chlorophyll a, b, carotenoids, and all total pigmentation), the treatment of ½ media power and 15 g/L sucrose is the most significant and highest one. In total protein, the highest yield treatment is ½ media with 5 g/L sucrose, which could be due to secretion of some stress proteins to enhance plant defense against sucrose deficiency. However, in total soluble sugars, the highest yield is in ¼ media power with 15 g/L sucrose. This may be a response to deficiencies in nutrients in supplemented media and as a defense mechanism.

This work was enhanced by [22], who applied different sucrose concentrations on the *in vitro* culture of *Ficus carica*. They approved that these concentrations improve the rooting of *Ficus*. Also, [23] proved that different sucrose concentrations enhance shooting in the in vitro culture of *Billbergia zebrina*.

Sucrose is essential in the growth medium for many plants' in vitro regeneration process [23, 24]. Nutrients (represented in media power) may become unavailable to the explants and lead to a restriction in their growth and, consequently, an increase in explant mass. Fresh and dry mass accumulation relates to nutrition [25]. Induction of adventitious root is related to the concentration and endogenous balance of nutrients composition and plant hormones [26].

Table 1. Effect of medium strength and sucrose and their interaction on fresh weight, shoot length, root length, leaf length, leaf number and root number of *P. alba* from *in vitro* rooting.

Sucrose (g/l)	**Medium strength**			**P-value**	**F-value**
	¼	½	¾		
Plantlet fresh weight (g)				**0.142**	**1.701**
0	0.1836 [a]	0.2205 [a]	0.4914 [a]		
5	0. 1870 [a]	0.2237 [a]	0.2004 [a]		
10	0.4569 [a]	0.3263 [a]	0.1785 [a]		
15	0.3785 [a]	0.5035 [a]	0.2444 [a]		
Shoot length (cm)				**0.000**	**5.500**
0	6.2233 [a, b, c, d]	4.7833 [c, d]	3.5000 [d]		
5	7.3666 [a, b, c]	5.2100 [b, c, d]	6.4333 [a, b, c, d]		
10	8.4400 [a, b]	7.5000 [a, b, c]	5.0333 [b, c, d]		
15	8.2900 [a, b]	8.6666 [a]	6.6333 [a, b, c, d]		
Root length (cm)				**0.208**	**1.489**
0	3.5666 [a]	4.4766 [a]	2.1000 [a]		
5	4.4066 [a]	4.0433 [a]	4.4666 [a]		
10	3.4000 [a]	4.3333 [a]	2.5666 [a]		
15	6.8850 [a]	4.1666 [a]	7.6000 [a]		
Leaf length (cm)				**0.462**	**1.022**
0	2.7333 [a]	3.1333 [a]	2.5500 [a]		
5	3.1333 [a]	3.2000 [a]	2.6666 [a]		
10	3.1000 [a]	2.7333 [a]	2.4666 [a]		
15	2.7500 [a]	3.4000 [a]	3.1333 [a]		
Leaf number				**0.067**	**2.117**
0	7.0000 [b, c]	7.0000 [b, c]	5.5000 [d]		
5	8.6667 [a]	7.0000 [b, c]	7.0000 [b, c]		
10	9.5000 [a]	7.6667 [b]	6.3333 [d]		
15	9.5000 [a]	8.0000 [a, b]	6.6667 [c, d]		
Root number				**0.011**	**3.182**

0	8.6667 [a]	6.0000 [a, b]	2.0000 [a, b]		
5	6.3333 [a, b]	9.3333 [a]	6.3333 [b]		
10	9.0000 [a]	8.0000 [a, b]	5.3333 [a, b]		
15	10.500 [a]	6.0000 [a, b]	6.6667 [a, b]		

Table 2. Effect of medium strength and sucrose and their interaction on pigmentations, total protein and total soluble sugars of *P. alba* from *in vitro* rooting.

Sucrose (g/l)	**Medium strength**			**P-value**	**F-value**
	¼	½	¾		
Chlorophyll a (mg/g)				**0.000**	**635.498**
0	2.5286 [h]	3.0313 [f]	3.3805 [e]		
5	3.5440 [d]	2.7083 [g]	3.9033 [c]		
10	3.4250 [e]	4.0436 [a, b]	3.9306 [b, c]		
15	3.4885 [d, e]	4.0823 [a]	4.0253 [a, b]		
Chlorophyll b (mg/g)				**0.000**	**618.416**
0	1.3673 [h]	1.4980 [g]	1.3710 [g, h]		
5	1.9373 [d, e]	1.4266 [g, h]	1.8160 [e, f]		
10	1.8650 [d, e]	2.9400 [b]	1.7006 [f]		
15	1.9470 [d]	3.1776 [a]	2.2256 [c]		
Carotenoids (mg/g)				**0.000**	**12746.29**
0	5.0583 [j]	7.0063 [f]	4.2300 [k]		
5	9.7996 [c]	5.6030 [i]	6.0667 [g]		
10	9.4550 [d]	10.670 [b]	5.8196 [h]		
15	9.8560 [c]	11.620 [a]	7.6150 [e]		
Total pigmentation (mg/g)				**0.000**	**5925.104**
0	8.9530 [i]	11.536 [g]	8.9815 [i]		
5	15.282 [c]	9.7356 [h]	11.786 [f]		
10	14.745 [d]	17.654 [b]	11.451 [g]		
15	15.296 [c]	18.880 [a]	13.866 [e]		
Total protein (mg/g)				**0.000**	**2290.634**
0	37.809 [g]	57.428 [b]	18.214 [i]		
5	46.190 [d]	73.000 [a]	4.8095 [k]		
10	50.500 [c]	24.952 [h]	10.952 [j]		
15	44.071 [e]	24.380 [h]	41.000 [f]		
Total soluble sugars (mg/g)				**0.000**	**7036.261**
0	194.160 [c]	119.483 [f]	84.7300 [h]		
5	203.273 [b]	85.9633 [h]	59.6366 [i]		
10	204.745 [b]	136.596 [e]	85.1800 [h]		
15	267.590 [a]	103.550 [g]	145.684 [d]		

3.2. Molecular Marker (RAPD-PCR)

Genetic variation of micro-propagated *Populus alba* plants has practical utility and commercial implications. In this work, we assessed fingerprinting profiles of the different treatments of culture regenerants using RAPD markers to confirm whether the plantlets were genetically stable or not. A total of 7 random RAPD primers were tested for initial screening; only 4 primers gave reproducible and clear bands. The number of scorable loci for each RAPD primer varied from 3 (Deca-10) to 8 (Deca-4) (Table 3). The 4 RAPD primers produced 22 distinct and scorable bands ranged in size from 151 to 1713 bp. The polymorphic bands ranged from 1 band (with Deca-10 and Deca-11) to 4 bands (with Deca-4), with a total polymorphism percentage of 38.33% from all primers. The polymorphism was detected during the RAPD analysis of *in vitro* raised plants (Fig. 1a–d). It was proven that the regenerated plants' uniformity was maintained, indicating variation in genetic stability among the clones.

Application of molecular analysis of *in vitro* regenerated plants has been well documented by many workers [27, 28, 29, 30]. Molecular analysis is an efficient and reliable screening technique for tissue culture-derived plants [31, 32]. Reliable monitoring of variability in DNA sequences of plants has been achieved using several PCR based molecular markers such as RAPD, ISSR, SSR and AFLP. The absence of genetic variation using RAPD has been reported in several cases, such as axillary bud proliferation of chestnut rootstock hybrids and almond plantlets [33, 34].

A dendrogram of 12 clones (Fig. 2) was constructed based on the genetic similarity matrix from Table (4). It was found that more or less, all some clones are near to each other, especially the following some clones' treatments (3,4), (6, 8) and (7, 10). In comparison, the dendrogram of these 12 some clones (Fig. 3) were constructed based on morphological, physiological, and molecular results. It was found that only 11 and 12 some clones treatments were outgroups from the others. These findings were agreed with results obtained from CAP covariance ordination (Fig. 4). Such differences could be attributed to the effect of media components on cellular behavior affected by media power and sucrose concentrations supplemented in MS media culture. The dendrogram application to estimate the genetic stability of plants was agreed with [35] who applied dendrogram to estimate the variation *Cyclanthus* due to different hormonal and media compositions.

The *in vitro* culture may induce cellular control loss, resulting in somaclonal variations [36]. These variations could be due to factors such as explant type, culture medium composition, culture duration, phyto-regulators, genotype, number of subcultures, or transfers. All these conditions are considered capable of inducing this variability *in vitro* culture [37, 38, 39, 40].

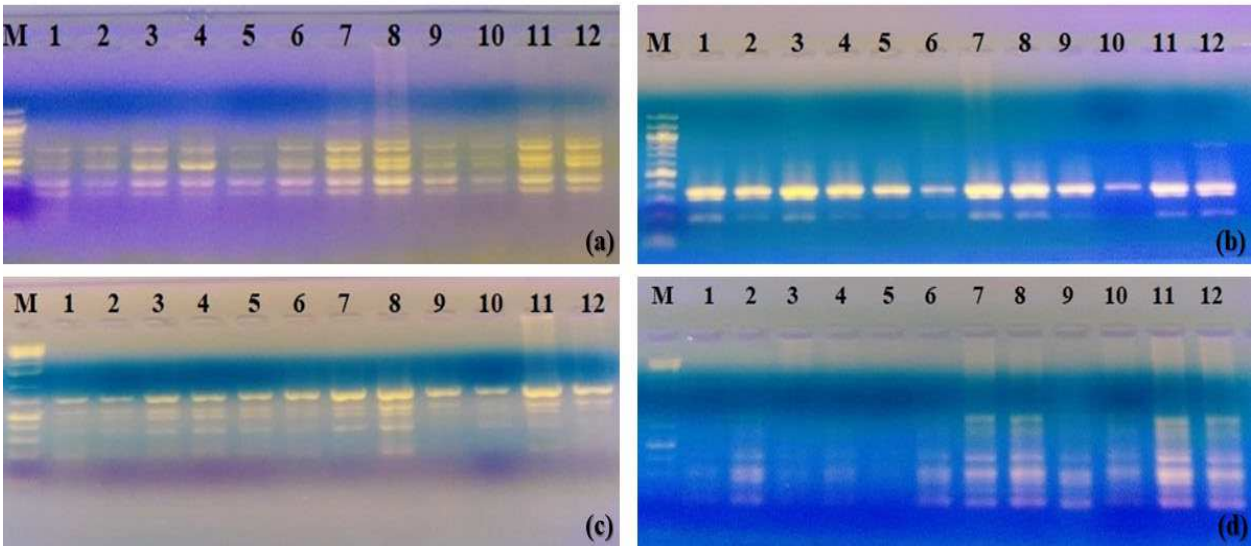

Fig. (1) Gel banding pattern of RAPD-PCR for *Populus alba* treatments. (a) Deca-4 primer, (b)Deca-10 primer, (c) Deca-11 primer and (d) Deca-13 primer.

Table (3) Primer Data analysis of RAPD-PCR bioassay with different *Populus* treatments

No.	Primer name	Primers sequence	GC%	Tm	Total bands	Size range (bp)	Total polymorphic bands	Polymorphism %
1	Deca 4	5'-CGTTGGCCCG-3'	80	44	8	235-1713	4	50
	Deca 10	5'-AGCCGGCCTT-3'	70	43.1	3	179-347	1	33.33
2	Deca 11	5'-ATCGGCTGGG-3'	70	39.3	5	206-619	1	20
4	Deca 13	5′-GTGGCAAGCC-3'	70	39	6	151-624	3	50
Total					22		9	38.33

Table 4 Total similarity matrix of different Populus treatments

	1	2	3	4	5	6	7	8	9	10	11	12
1	**100**	71.8	67.6	72.5	66.5	71.9	61.2	73.5	69.9	68.4	62	59.2
2	71.8	**100**	77.1	82.2	68.4	74.2	67	79.6	71.5	71.7	75.9	66.5
3	67.6	77.1	**100**	79.1	62.1	67	59.8	66.8	62.7	62.3	52	62
4	72.5	82.2	79.1	**100**	66	61.2	58.6	67.9	64.3	63.7	65.3	57.8
5	66.5	68.4	62.1	66	**100**	75	62	69.3	60.2	72.1	62.8	65.7
6	71.9	74.2	67	61.2	75	**100**	72.1	78.2	76.2	77.8	65.9	68.1
7	61.2	67	59.8	58.6	62	72.1	**100**	74.4	67.7	80.7	61.8	66.6
8	73.5	79.6	66.8	67.9	69.3	78.2	74.4	**100**	73.4	76.9	71.6	69.4
9	69.9	71.5	62.7	64.3	60.2	76.2	67.7	73.4	**100**	77.3	66.9	72
10	68.4	71.7	62.3	63.7	72.1	77.8	80.7	76.9	77.3	**100**	66.4	78.8
11	62	75.9	52	65.3	62.8	65.9	61.8	71.6	66.9	66.4	**100**	63.6
12	59.2	66.5	62	57.8	65.7	68.1	66.6	69.4	72	78.8	63.6	**100**

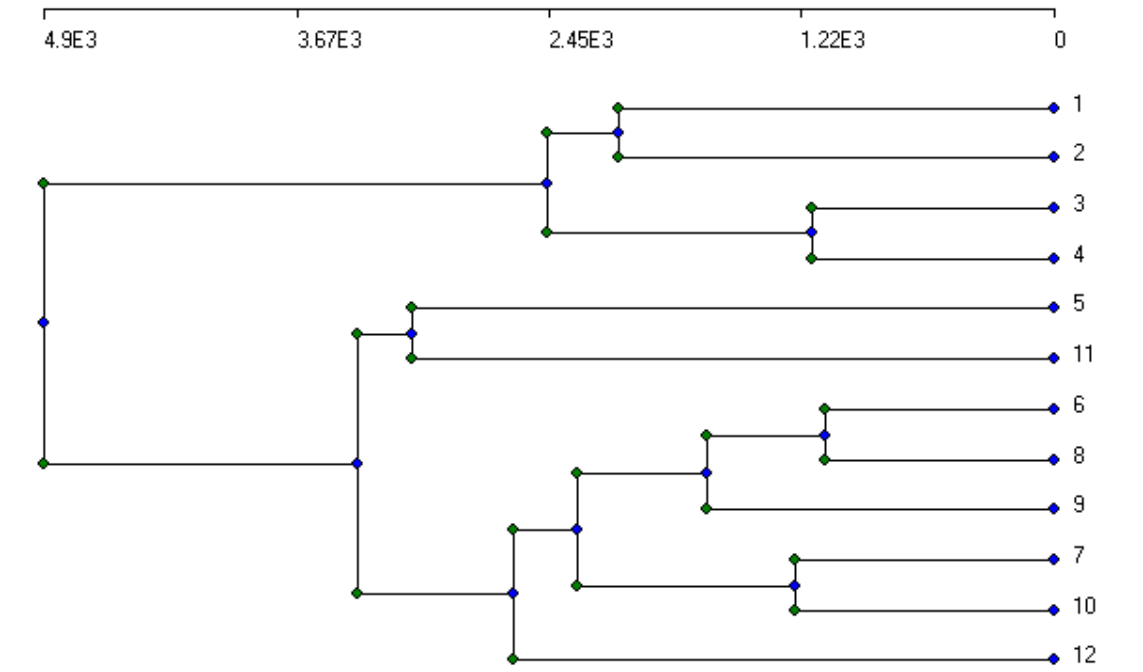

Fig. 2 UPGAMA phylogenetic tree of different *Populus* somaclones treatments based on RAPD-PCR data

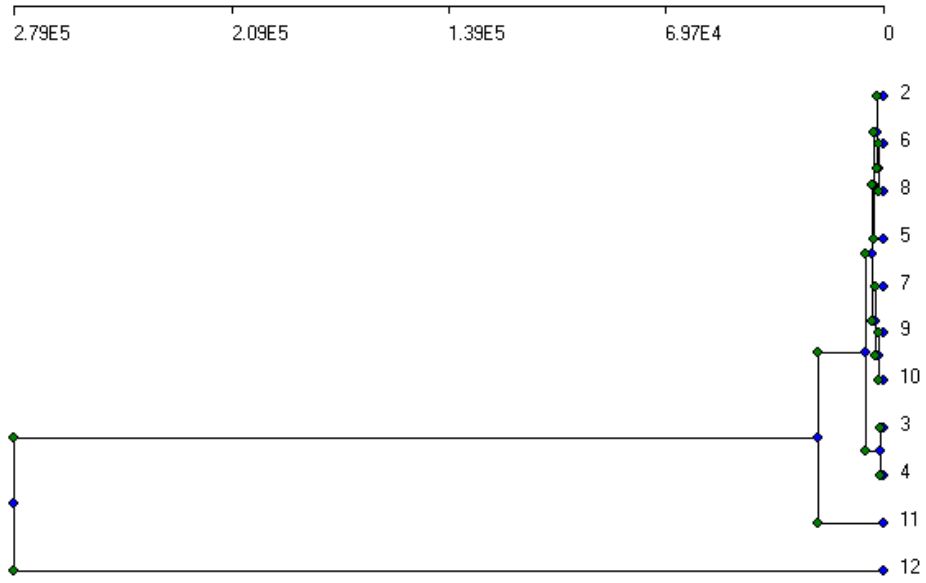

Fig. (3) The total complete linkage clustering analysis of combined morphology, physiology and RAPD-PCR responses resulted from different treatments of *P. alba*

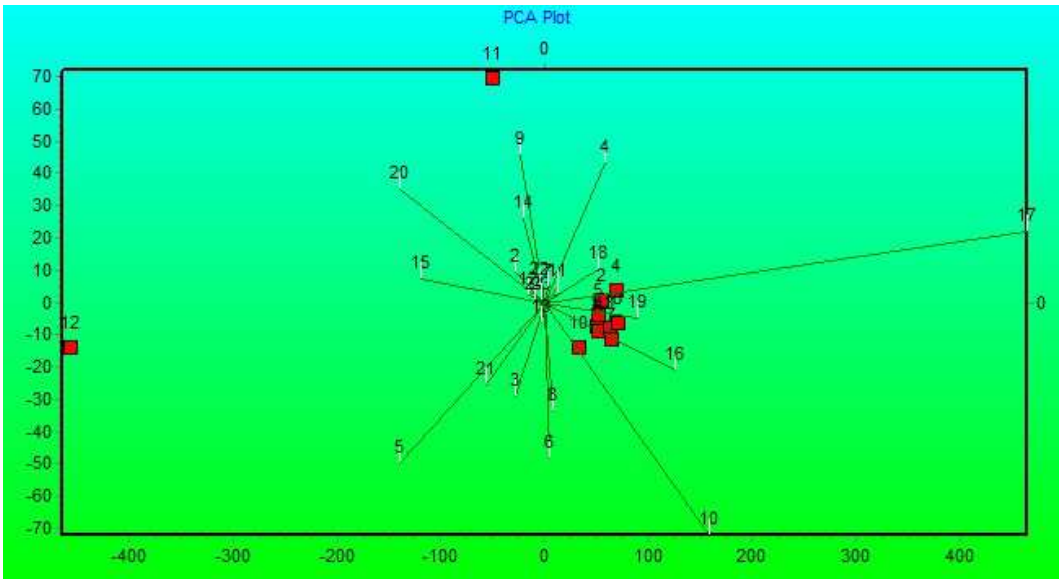

Fig. (4) PCA blot of PCA covariance ordination based on morphology, physiology and RAPD-PCR between different treatments of *P. alba.*

Conclusion

The present study describes an efficient protocol to control *Populus alba* micropropagation. The parameters, shoot length, leaves, and root numbers, affected by the interaction between sucrose and minerals levels (in media composition), which is useful in micro-propagation. *P. alba* responding to different media composition led to soma clonal variation among the micro propagated plants. The genetic variation resulting from this media isn't so high (38.33%), which ensures some genetic stability within some clones of *P. alba*.

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Figures

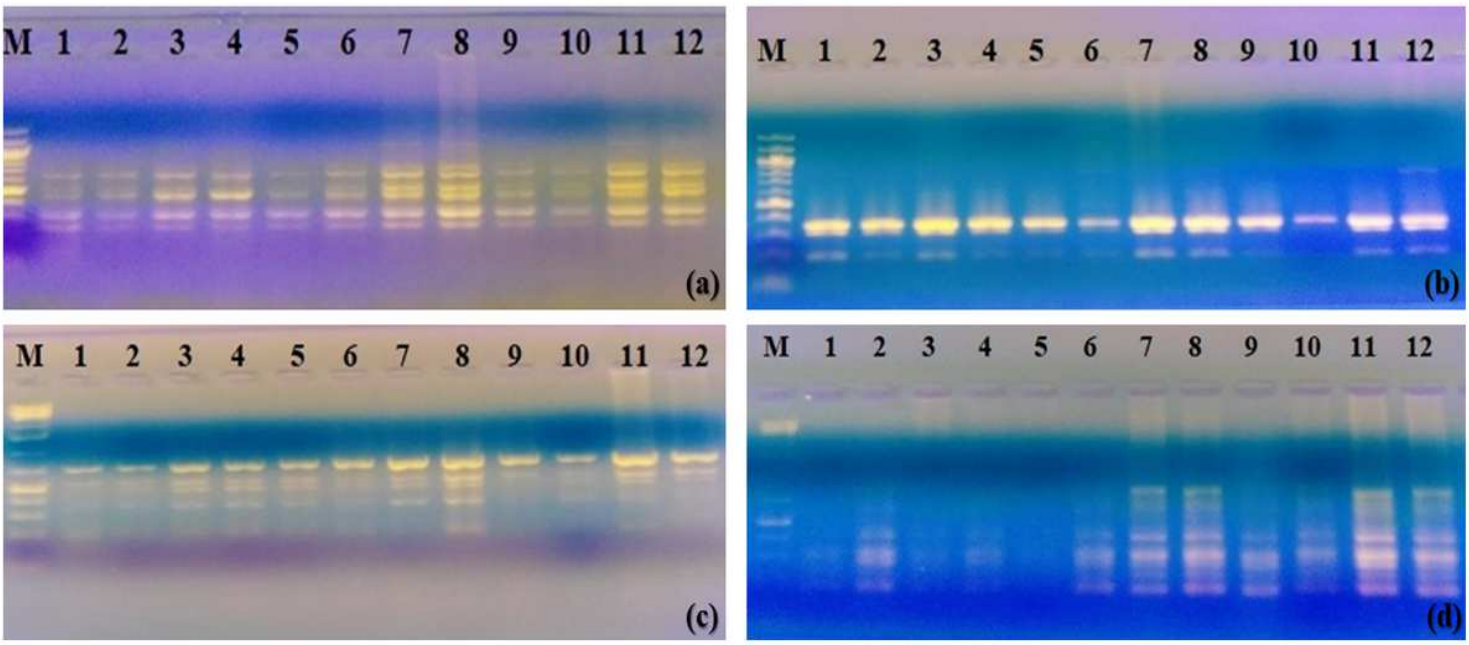

Figure 1

Gel banding pattern of RAPD-PCR for Populus alba treatments. (a) Deca-4 primer, (b)Deca-10 primer, (c) Deca-11 primer and (d) Deca-13 primer.

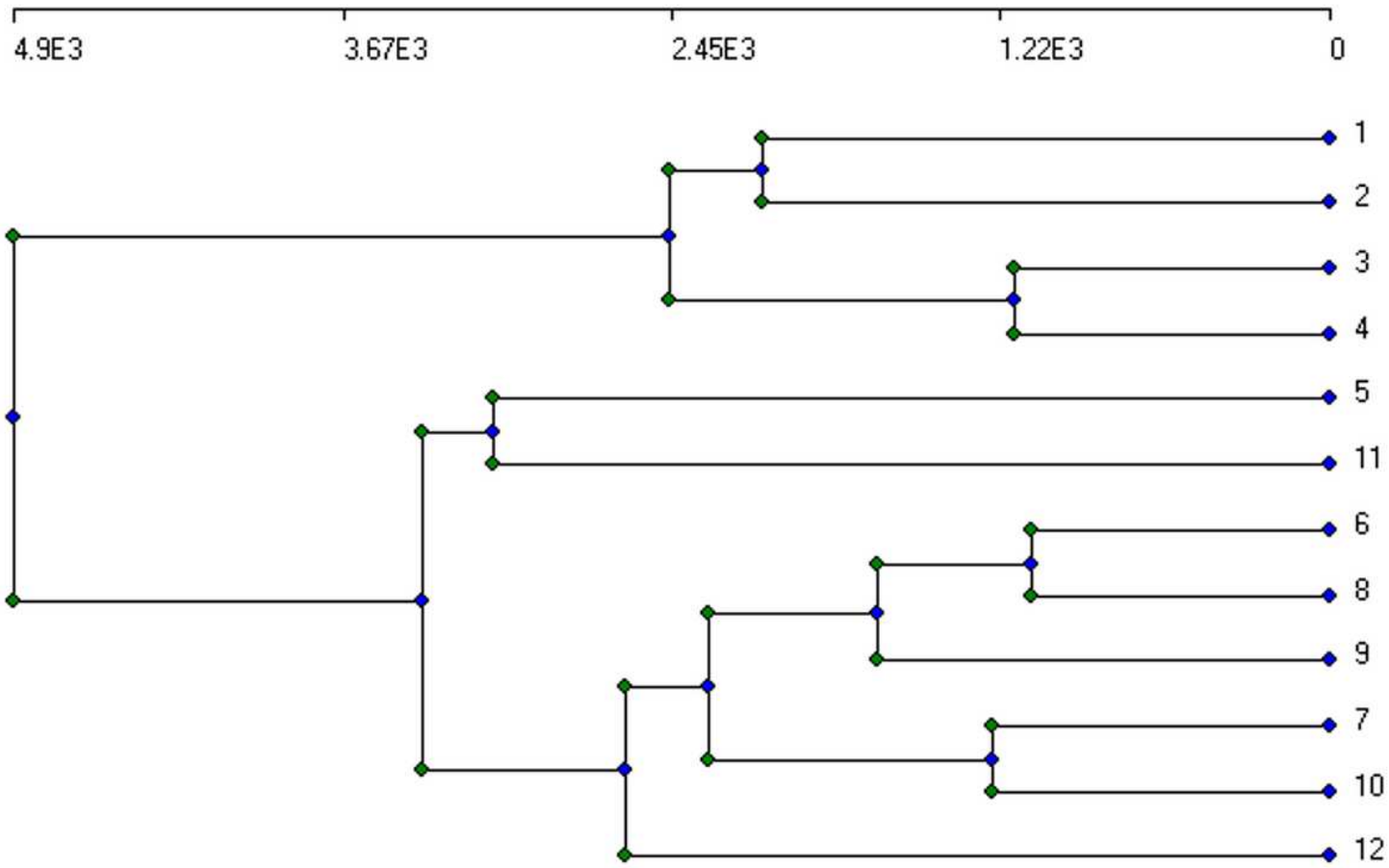

Figure 2

UPGAMA phylogenetic tree of different Populus somaclones treatments based on RAPD-PCR data

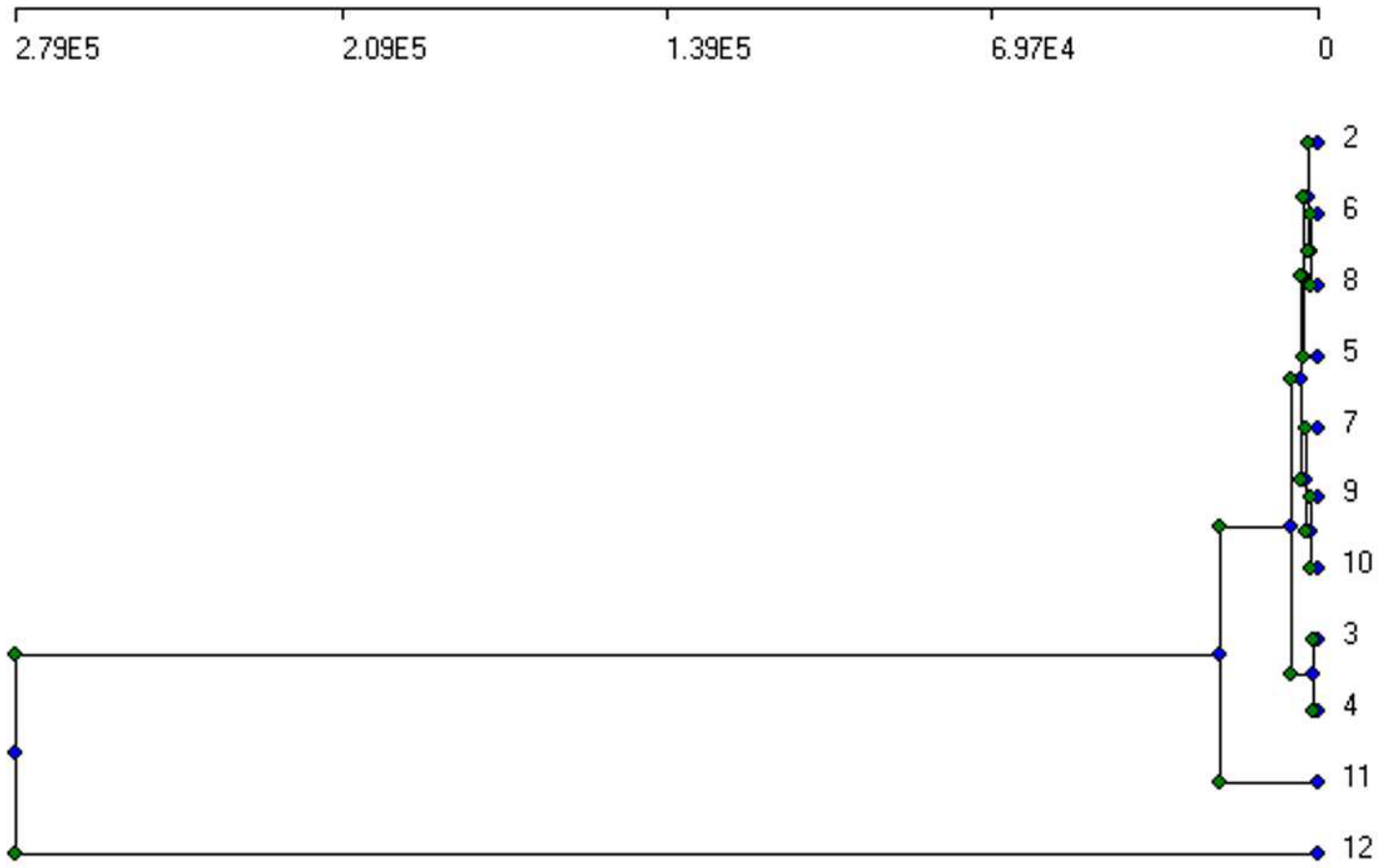

Figure 3

The total complete linkage clustering analysis of combined morphology, physiology and RAPD-PCR responses resulted from different treatments of P. alba

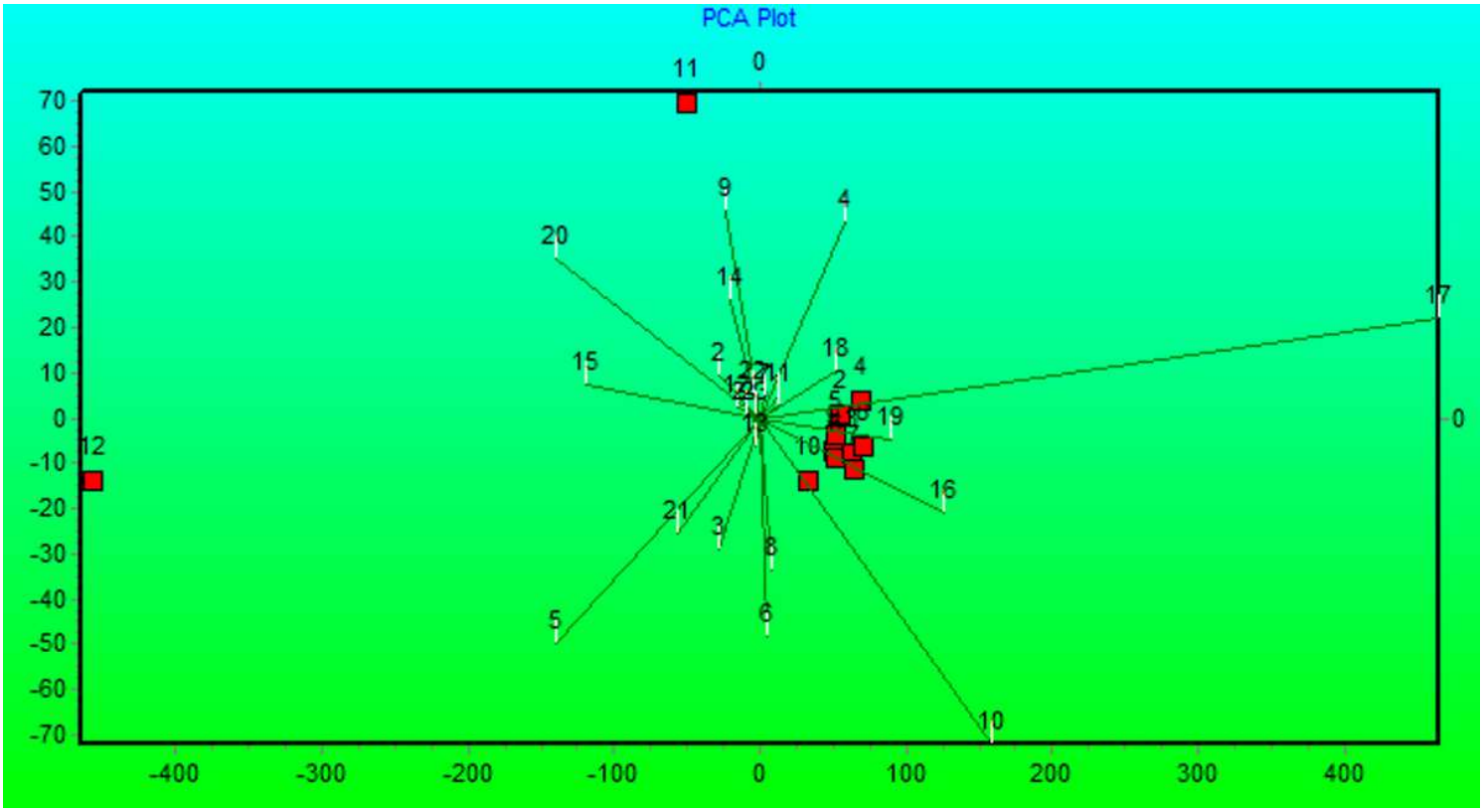

Figure 4

PCA blot of PCA covariance ordination based on morphology, physiology and RAPD-PCR between different treatments of P. alba.