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Genetic Stability Assessment of *in Vitro* Rooted *Populus Alba* L. Micro-Shoots in Different Media compositions

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Abstract

Populus alba is a huge woody plant. The bark is very white and smooth when the tree is young. With age, the bark becomes darker and more furrowed. This work manipulate the idea of variation of the new individual resulted from plant tissue culture due to any change in media composition. Previously it was known that all the new resulted individuals are genetically similar, but in this study, the contrast was proved. As the new individuals are genetically different. The plant was multiplied in shooting and rooting MS media. This study was designed based on two parameters (media power and hormone concentrations) resulting in 25 treatments. It was known that the new plantlets from tissue culture were identical, similar to the source mother plant and each other as well. Nevertheless, the current study manipulated the effect of various media power and auxins concentrations on *P. alba's* molecular responses. Hence, the genetic stability of the resulted new individuals from treatments is completely different. Also the different auxin hormone concentration (IBA and NAA) made genetic instability in the new resulted individuals. A molecular marker (RAPD-PCR) was used to estimate the genetic variations. Four decamers yielded sorable bands and led to a total polymorphism percentage of 48.26. The morphological and physiological variation originated from the genetic variation these results confirm that the new resulted plantlets from different tissue culture conditions could be different from each other and led to different growth parameters.

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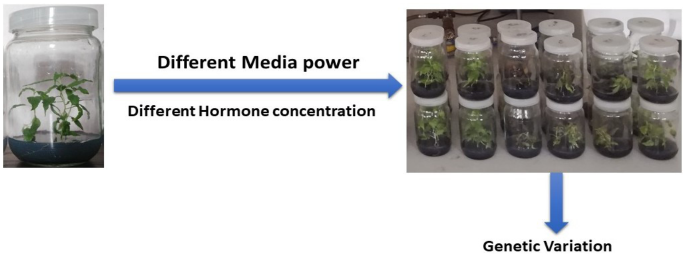
Graphical abstract



Introduction

The genus *Populus* is widespread all over the world, especially in the Northern part of Earth. They are dioecious, a medium-sized woody tree with simple, glabrous leaves and buds covered with scales. It belongs to the family *Salicaceae* [1,2]. The genus *Populus* is the main model for physiological, molecular genetic studies in trees [3]. *Populus spp* are important pattern for woody perennial biotechnology because it can change genetic engineering through *Agrobacterium*-gene transformation and *in vitro* culture [4]. It was the first tree in which the genome was sequenced [5]. White poplar (*Populus* alba) is a native to the Mediterranean geographical area. *Populus* is a deciduous and fast-growing tree. White poplar leaves are applied as biomonitors for soil pollution [6].

Populus alba was a main and essential objectives for *in vitro* propagation trials. *Populus* culture's explants started as follows: cambial, callus improvement, followed by shoot or root developments. The previous works concluded that the vegetative propagation steeled from different originated callus-based plant regeneration. Initially, there were obstacles to culture establishment and genetic determination differences among the species. The success of the settlement depends on the mother plants age. Recently, the development of poplars micropropagation protocols is for commercial purposes with media optimization. The breeding study relied on *in vitro* explants started with developing an *in vitro* mass-propagation procedure of *Populus*. This protocol based on protoplast and cell suspension productions followed by plant regeneration [7].

Gifston et al. [8] mentioned that phenolic compounds has also an essential role in the plant antioxidant system. Furthermore, Amin et al. [9] and Gad El-Hak et al. [10] revealed that phenolic compound stimulated vegetative growth, protein content, total carbohydrate, nitrogen, phosphorus, potassium and yield of different plants.

There are several molecular markers used for differentiation among species and populations. These markers, like Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP). They were employed to estimate the frequency of genomic polymorphism among different transformed plant lines in comparison to no transgenic lines [11,12]. Additionally, they could be used for detecting polymorphism at the DNA level. For example, RAPD-PCR gained much popularity for its simplicity and doesn't require prior information on the nucleotide sequence. RAPD-PCR can be implemented with a tiny set of genomic DNA. RAPD technique is efficient, simple, reliable, and an cost-effective means of cultivar identification and diversity analysis [13].

In various studies, the genetic diversity of some plants has been investigated using different molecular markers. The study of molecular variability and phylogenetic relationships, varietal identification, gene map-based cloning or Quantitative Trait Loci, are the most important uses (QTLs). Despite using different molecular markers to examine genetic diversity in cultivated plant species, many identify a limited level of polymorphism. Thus, the identification of more polymorphic molecular markers is important for research [14].

Phenotypic, physiological and genetic variations occur due to micro-propagation process with various media constituents. Hence, it is essential to estimate the genetic stability of *Populus* alba. This study monitors the genetic variation and unstability of long-dated micro-propagated shoots of *P. alba* by RAPD-PCR molecular technique. None of these studies have previously been investigated for *P. alba in vitro* propagation.

Materials and methods

Collection of plant materials

Populus alba nodal explants were obtained from the Horticulture Research Station, Al-Gharbia Governorate. Afterthat, segments were cultured in tissue culture laboratory in Agriculture Center for Genetic Engineering and Biotechnology (ACGEB), in Faculty of Agriculture, Ain Shams University, Egypt).

Establishment of explants

The explants were Scratched and cleaned carefully with tap water to erase all dust particles. The explants followed by surface sterilization using 20% Clorox + 0.1% $HgCl_2$ for 20 mins and washed four to five times with steriled ddH_2O. Afterthat, nodal segments of *P. alba* were sterilized then cultured for two months on free MS media supplemented with required macro- and micro-nutrients (Caisson, MSP09-50LT) as described by Murashige and Skoog [15] with 6g agar. Total of 60ml of medium was placed into incubation jars. The cultured stem nodal segments were incubated at about 25°C and supplied with white fluorescent light (3000 Lux/ 16-hour photoperiod/ cooling).

In vitro rooting experiment design

After stem nodal segments of *Populus alba* were maintained on MS tissue culture media, the roots were manipulated. For root formation, the developed shoots were transferred into MS multiplication media with activated charcoal (0.5 g/L), low levels of 6-Benzylaminopurine (BAP) and sucrose (20 g/L) was then transferred and cultured in 400ml jars with 60ml MS media. Resulted shoots from the establishment were excised and transferred into the multiplication medium of MS provided with 0.075 mg/L of BAP to acquire micro-shoots required for the rooting experiment. The jars were incubated at 25±2 °C for five weeks (16 h light/8 h darks).

The experiment was designed as follow: different media powers were prepared (1/8, ¼, ½, ¾ and full MS) and different hormone concentration (0, 0.1 IBA, 0.5 IBA, 0.1 NAA and 0.5 g/L NAA). The equivalent weights of different media power were as follow: 1/8 MS equal to 0.55 MS in 1L dH_2O, ¼ MS equivalent to 1.1 g/L, ½ MS equivalnt to 2.21 g/L, ¾ MS eqivalent to 3.32 g/L and full MS eqivalent to 4.43 g/L.

DNA Isolation and RAPD-PCR Bioassay

The total genomic DNA of *Populus alba* individuals was extracted by CTAB method, according to Doyle and Doyle [16]. Half gram of leaves was mixed with 800 µl of 2% CTAB buffer, then incubated 45mins at 65 °C (vortex each 10mins). Centrifuge tubes at 12,000 rpm for 12 mins, then transfer the supernatant into new tubes with addition of equal volume of chloroform: isoamyl alcohol (24:1) and set for 3mins at room temperature. Afterthat tubes were centrifuged (12,000 rpm / 10 mins / 4°C). Then the upper aqueous layer was transferred to new Eppendorf tubes with addition of 800 µl of absolute ice-cold ethanol and left overnight at -20 °C. Tubes were centrifuged to precipitate DNA pellets then washed them with ice-cold 70% ethanol. Finally, resuspend pellets in 50 µl of TE buffer and keep at -20 °C till applying RAPD-PCR.

Seven decamers were applied in this study, however only four of them yielded scorable and reproducible bands (Table 2). The RAPD-PCR reaction was carried out in Biometra thermocycler with a total volume of 25 µl of 12.5 µl Taq master mix (COSMO PCR RED M. Mix, W1020300x), 3 µl of genomic DNA, 1.5 µl for each primer (Willowfort) and 8 µl ddH_2O. The reaction program designed as 40 cycles as follow: Denaturation for 30 sec at 94 °C, annealing 30 sec at different degrees (Table 2) and extension for 1min at 72 °C; following by one step of final extension at 72 °C for 10mins then cooling at 4 °C. The amplified PCR product was run on 1.4% agarose gel compared to (New England Biolab, #N3232S) ladder.

Statistical analysis

The analysis of gel electrophoresis resulted in images that were analyzed by band scoring (1,0) and a pairwise similarity matrix was generated using Jaccard's similarity coefficient, and using the Unweighted Pair Group Method with the Arithmetic Averaging Algorithm (UPGMA). These computations were carried out using Bio-Rad Quantity one (4.6.2) and Community Analysis Package (CAP, 1.2) [17].

Results

Tissue culture and plantlets morphology

The resulted plantlets of *P. alba* from the different treatments show significant differences among them. Some of these treatments were illustrated in Figure (1). For indication, the different treatments were shown in Table (1).

Table 1: The different treatments of media power and growth hormones.

No.	Treatment	No.	Treatment
1	1/8 MS + 0 hormones	14	1/2 MS + 0.1 NAA
2	1/8 MS + 0.1 IBA	15	1/2 MS + 0.5 NAA
3	1/8 MS + 0.5 IBA	16	3/4 MS + 0 hormones
4	1/8 MS + 0.1 NAA	17	3/4 MS + 0.1 IBA
5	1/8 MS + 0.5 NAA	18	3/4 MS + 0.5 IBA
6	1/4 MS + 0 hormones	19	3/4 MS + 0.1 NAA
7	1/4 MS + 0.1 IBA	20	3/4 MS + 0.5 NAA
8	1/4 MS + 0.5 IBA	21	Full MS + 0 hormones
9	1/4 MS + 0.1 NAA	22	Full MS + 0.1 IBA
10	1/4 MS + 0.5 NAA	23	Full MS + 0.5 IBA
11	1/2 MS + 0 hormones	24	Full MS + 0.1 NAA
12	1/2 MS + 0.1 IBA	25	Full MS + 0.5 NAA
13	1/2 MS + 0.5 IBA		

Molecular marker

RAPD is a PCR-based molecular technique, simple and require only small quantities of DNA samples. In this study, the reproducible 4 RAPD primers were: Deca-4, Deca-11, Deca-12 and Deca-13. These primers gave a total number of 26 bands for *P. alba.* The total polymorphism percentage was 48.26%. These primers' data in detail were illustrated in the Table (2) and Figure (2). The total similarity matrix resulted from all these four primers were indicated in the Table (3) to show the rate of similarity among the different of *P. alba* treatments. It was calculated as an average of the four similarity matrices resulted from the reproducible 4 RAPD primers. The dendrogram resulted from RAPD data was represented in Figure (3). The dendrogram illustrated genetic similarities among some treatments like 1/8 MS + 0.5 NAA and 1/4 MS + 0.1 IBA; ½ MS with both 0.5 IBA and 0.1 NAA; and 3/4 MS with both 0.5 IBA and 0.1 NAA.

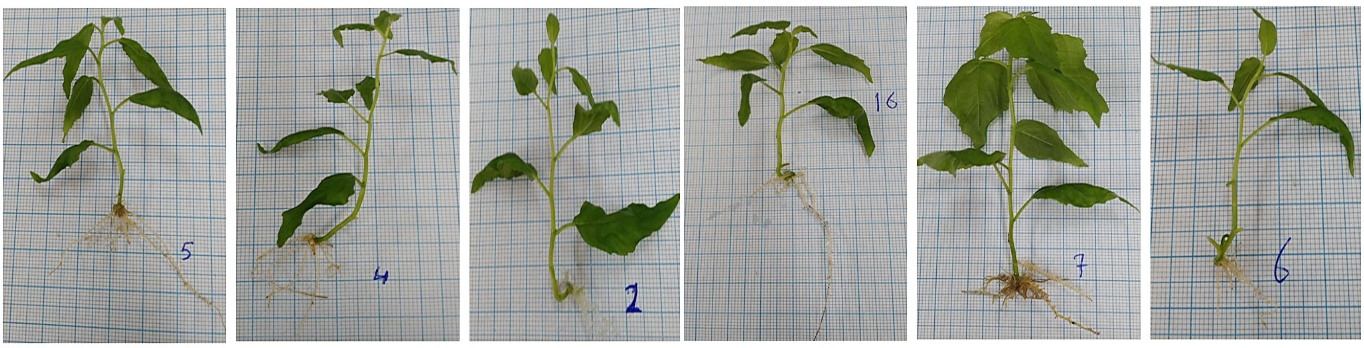


Figure 1: The morphology of different treatments of *P. alba.*

Table 1: Primer Data analysis of RAPD-PCR bioassay with different *Populus* treatments.

No.	Primer name	Primers sequence	GC%	Tm	Total bands	Total polymorphic bands	Polymorphism %
1	Deca 4	5'-CGTTGGCCCG-3'	80	44	9	5	55.55
2	Deca 11	5'-ATCGGCTGGG-3'	70	39.3	8	3	37.5
3	Deca 12	5'-CTTGCCCACG-3'	70	38.5	3	2	66.67
4	Deca-13	5'-GTGGCAAGCC-3'	70	39	6	2	33.33
Total					26	12	48.26

Table 2: Total similarity matrix of all primers with different *Populus* treatments.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	**100**	51	73	73	79	59	78	72	44	43	67	71	77	72	65	58	66	70	71	68	67	61	63	63	58
2	51	**100**	70	66	56	61	66	64	48	69	64	63	65	59	62	58	61	62	62	65	56	53	55	69	65
3	73	70	**100**	66	66	54	43	69	76	68	57	58	68	62	66	55	56	65	67	79	47	58	53	65	55
4	73	66	66	**100**	73	62	75	75	63	68	61	70	73	68	66	58	64	67	62	57	58	60	54	59	52
5	79	56	66	73	**100**	60	77	72	67	64	60	72	68	69	67	60	66	71	70	59	73	76	62	69	58
6	59	61	54	62	60	**100**	55	71	58	52	51	72	57	54	59	65	62	70	68	52	55	53	50	60	54
7	78	66	43	75	77	55	**100**	79	68	74	67	74	81	77	71	63	71	71	72	63	73	67	66	72	60
8	72	64	69	75	72	71	79	**100**	72	71	70	74	75	69	74	68	76	85	83	66	68	64	59	69	58
9	44	48	76	63	67	58	68	72	**100**	67	59	57	61	61	60	67	61	68	66	66	63	68	64	72	62
10	43	69	68	68	64	52	74	71	67	**100**	58	60	70	68	78	50	60	64	63	67	58	63	61	70	64
11	67	64	57	61	60	51	67	70	59	58	**100**	74	59	52	64	64	66	72	71	56	65	64	65	70	67
12	71	63	58	70	72	72	74	74	57	60	74	**100**	69	66	68	72	72	70	73	56	69	48	60	69	59
13	77	65	68	73	68	57	81	75	61	70	59	69	**100**	83	73	53	64	71	75	64	64	56	60	61	47
14	72	59	62	68	69	54	77	69	61	68	52	66	83	**100**	61	53	64	68	73	60	61	58	56	58	51
15	65	62	66	66	67	59	71	74	60	78	64	68	73	61	**100**	55	58	67	67	64	64	42	57	70	62
16	58	58	55	58	60	65	63	68	67	50	64	72	53	53	55	**100**	73	61	62	52	71	57	59	64	60
17	66	61	56	64	66	62	71	76	61	60	66	72	64	64	58	73	**100**	75	72	56	69	59	59	62	48
18	70	62	65	67	71	70	71	85	68	64	72	70	71	68	67	61	75	**100**	83	64	67	66	60	65	59
19	71	62	67	62	70	68	72	83	66	63	71	73	75	73	67	62	72	83	**100**	67	65	64	57	66	61
20	68	65	79	57	59	52	63	66	66	67	56	56	64	60	64	52	56	64	67	**100**	56	54	51	65	60
21	67	56	47	58	73	55	73	68	63	58	65	69	64	61	64	71	69	67	65	56	**100**	68	74	71	62
22	61	53	58	60	76	53	67	64	68	63	64	48	56	58	42	57	59	66	64	54	68	**100**	63	69	71
23	63	55	53	54	62	50	66	59	64	61	65	60	60	56	57	59	59	60	57	51	74	63	**100**	70	54
24	65	69	65	59	69	60	72	69	72	70	70	69	61	58	70	64	62	65	66	65	71	69	70	**100**	74
25	58	65	55	52	58	54	60	58	62	64	67	59	47	51	62	60	48	59	61	60	62	71	54	74	**100**

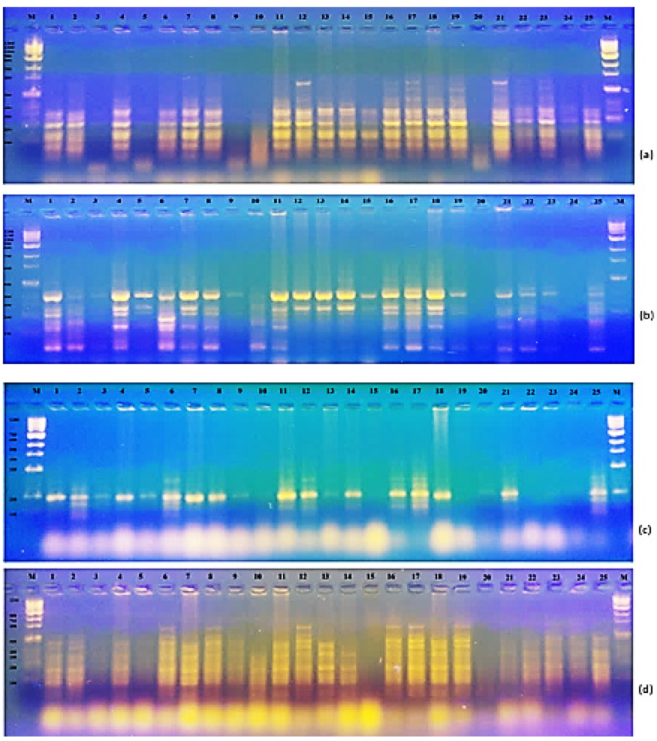


Figure 2: RAPD-PCR profile gel electrophoresis for the different treatments of *Populus* alba. (a): with Deca 4 primer; (b): with Deca 11 primer; (c): with Deca 12 primer and (d): with Deca 13 primer.

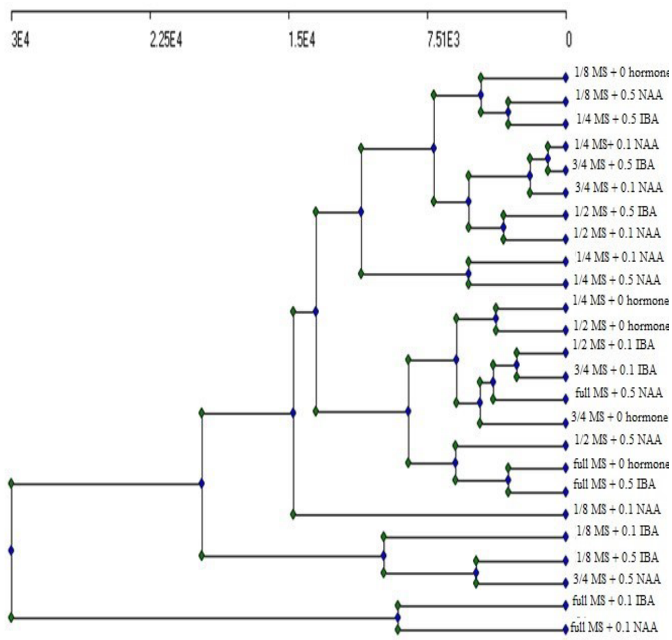


Figure 3: The total complete linkage clustering analysis of RAPD-PCR responses resulted from different treatments of *P. alba.*

Discussion

As the use of micro-propagated plantlets has become increasingly popular, genetic uniformity issues are emerging. Therefore, morphological description, physiological measurements and molecular assessments were performed to assess the magnitude of change in the structure of plants resulted from response to change in growth media constituents. The lower IBA concentration showed a better performance per young rooted shoots on root number and length than NAA. These findings agreed with the outcome of Khattab's et al. [18] on *P. alba.* Depending on Hewidy et al. [19], there was significant variations in these treated plants' morphological and physiological parameters.

Genetic stability in the regenerated plants is vital for species conservation and fidelity [20]. To confirm if the soma-clonal variation in the regenerated individuals, RAPD was applied to analyze the genetic stability of plant species from randomly *in vitro* derived plants and control donor plants.

The polymorphism percentage and variation in the propagated plants' genetic stability could be explained for many reasons. Such reasons are (1) Different applied hormones and their concentrations as explained by Werner et al. [21] and Kasim et al. [22], who used different hormones at various concentrations which proved that the possibility of genetic instability recorded by RAPD marker and demonstrated significant variation in plant morphology. (2) Different media strength was explained by Gnamien et al. [23], who used different strength of media compositions and sucrose concentrations. (3) Different sucrose levels in media composition. Or (4) long incubation period, the normal incubation period ranges from 21 to 28 days while these plantlets were incubated for seven weeks (about 50 days); this agreed with Lakshmanan et al. [24], who used both ISSR and RAPD markers to assess the genetic variation/stability in long-term regenerated shoots of the banana plant. Also it agreed with Werner et al. study [21], who estimated the genetic stability in *Crambe abyssinica* plant along different incubation periods and found little variation in genetic content using the ISSR marker. According to previous studies, they seem to manipulate one or two factors only to study incubated plants' response and their genetic stability of plants. However, in this study many different factors were combined to prove that any change in media composition, hormones type or even concentration may lead to genetic instability and polymorphism.

However, other studies examined the changes in media composition and realized genetic stability without changed between the regenerated plants and the mother plants. Soni and Kaur [25] have proved the stability in regenerated plantlets' genetic content resulted from *in vitro* propagated *Viola pilosa* with different hormones at different concentrations. Besides, Saha et al. [26] used RAPD and ISSR molecular markers to prove that there was genetic stability in *Morus alba* with the different growth hormones with different concentrations. Furthermore, Oliveira et al. [27] present the plants' genetic stability from different plantation cutting techniques (mini-cutting, micro-cutting and *in vitro* culture). Also, Goda et al. [28] proved the importance of having a stable platform for the conservation of endangered *Capparis spinosa* with high genetic fidelity even under different growth regulators.

We can explain the argument in different growth hormones upon the growing stage in the *in vitro* culture, where it could cause genetic stability or instability due to the different types of growth hormones. According to the rooting stage hormones, both NAA and IBA were studied. At the same time, other studies were concerning the multiplication stage with the application of various cytokinins, i.e. BA, BAP, Kin, 2-IP and TDZ [15,26,28].

In general, it is essential to confirm that the regenerants in genetic fidelity are genetically true-to-type of their donor plants. The RAPD marker system has been used for this target to know where there is an aberration in the regenerated plants. This scheme has been shown to be a potential marker for the distinction between genetic variation and genetic fidelity of popular micro-propagated plantlets [29, 30, 31]. These results confirm that in our regeneration system, all of the regenerants showed genetic stability. Therefore, it could be concluded that no somaclonal variation shown in other cultures mediated by other explants was induced by direct regeneration from shoot tip explants [32,33].

Conclusion

There is a highly visualized point clear from this work: the new plants resulting from the mother plant are not genetically identical as they could be affected by any change in the MS media component. This change is illustrated here and concluded from the high polymorphism percentage resulted from the RAPD-PCR reaction. The polymorphism percentage is high and could reach to 48.26% due to change in media components. This proves that the plants are very highly sensitive to any change in salts or hormones concentration. The further work will manipulte the effect of many different factors on media and growth hormones on the general behavior of plant.

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