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Molecular Genetic Markers for Tissue Culture Response in Garlic (*Allium sativum* L.)

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Abstract

Five genotypes of garlic (*Allium sativum* L.), namely Balady (G1), Chinese (G2), Sids-40 (G3), Egaseed-1 (G4) and Egaseed-2 (G5) were used for assessing tissue culture response, genetic variability and polymorphism amongst which and to identify molecular markers associated with tissue culture response using ISSR and SRAP techniques. Significant differences were found among the five genotypes, the concentration of growth regulators as well as the interaction between them for all measured tissue culture traits. The Euclidean-distance dendrogram using tissue culture data separated the five garlic genotypes into two clusters; the first cluster comprised the highly responsive genotypes (G1, G2 and G3) while the less responsive genotypes (G4 and G5) grouped together in the second cluster. Using two molecular marker systems (ISSR and SRAP), a total of 191 fragments were amplified from the five garlic genotypes and 107 (56.61%) of them were polymorphic. The dendrogram generated based on combined ISSR and SRAP data showed two main clusters, the first comprised of one genotype (G2) which had the highest mean values for three tissue culture traits on all concentrations of growth regulators, while the second comprised the four other garlic genotypes (G1, G3, G4 and G5) which were the less responsive. Highly significant positive correlation ($r = 0.595$: $p = 0.001$) was found between the data of the tested molecular markers and tissue culture response. Thirteen DNA fragments were found to be positive molecular genetic markers for tissue culture response in garlic genotypes. The investigation demonstrated that ISSR and SRAP analyses showed considerable potential for variety identification and discrimination and could be useful for tissue culture response in garlic.

Keywords*: garlic, genetic variability, polymorphic*

Introduction

Garlic (*Allium sativum* L.) is a monocot species and belongs to the family Liliaceae and genus Allium, which contains more than 600 species (Osman *et al.,* 2007). Garlic has been cultivated and consumed worldwide since ancient Egyptian period. Garlic bulb is an important seasoning ingredient in many of the world's cuisines, and the green flower stalks and young leaves are eaten fresh or cooked.

Furthermore, large quantities of garlic are used for pharmaceutical purposes (Kik and Gebhardt, 2001), and garlic extract has been used as a traditional medicine for the prevention and treatment of cardiovascular disease (Ackermann *et al.,* 2001).

Tissue culture protocols are efficient tools for complementing traditional garlic breeding programs and producing cultivars with higher yields, higher tolerance/resistance to viral and fungal diseases and better adapted to local environmental conditions (Barandiaran *et al.,* 1999a, b, c; Martı´n-Urdı´roz *et al.,* 2004).

Several factors affecting plant regeneration in garlic such as the ex-

plant type, the physiological condition of the explant, the genotype and the growth regulator combination used in the culture medium (Abo El-Nil, 1977; Myers and Simon, 1998; Barandian *et al.,* 1999a, b, c; Robledo-Paz *et al.,* 2000; Parvin *et al.,* 2007; Nadeem *et al.,* 2017)

Diversity in plant genetic resources (PGR) provides opportunity for plant breeders to develop new and improved cultivars with desirable characteristics, which include both farmer-preferred traits (yield potential and large seed, etc.) and breeders preferred traits (pest and disease resistance and photosensitivity, etc.). Traditional methods for evaluating garlic diversity rely on resolving differences in morphological characters. However, the information provided by this approach is limited since the expression of such characters may differ under varying environmental conditions. Because of phenotypic flexibility and the occurrence of mutations, the identification and systematic classification of garlic is difficult.

In recent years, molecular markers such as random amplified polymorphic DNA (RAPD) (Ipek *et al.,* 2003 ; Khar *et al.,* 2008), amplified fragment length polymorphisms (AFLP) (Morales *et al.,* 2013), SSR (Cunha *et al.,* 2012; Ma *et al.,* 2009), sequence-related amplified polymorphism (SRAP) (Chen *et al.,* 2013) , inter-simple sequence repeat (ISSR) (Jabbes *et al.,* 2011; El Nagar and El-Zohiri, 2015; Gehan *et al.,* 2017; Rakesh *et al.,* 2018) have been used to assess genetic diversity and the relationships among garlic varieties, as they are not affected by environmental conditions (Jo *et al.,* 2012). These markers are extremely sensitive and are capable of identifying allelic germplasm collected from the different geographical regions of the world.

The objectives of the present investigation were; (1) to study tissue culture response and plant regeneration in garlic (*Allium sativum* L.); (2) to study genetic variability and polymorphism among five garlic genotypes using ISSR and SRAP markers, (3) to identify markers associated with tissue culture response.

Materials and Methods

This study was carried out at the tissue culture laboratory, Vegetables department and Biotechnology laboratory, Genetics department, Faculty of Agriculture, Assiut University during 2016 – 2018.

Five genotypes of garlic (*Allium sativum* L.), namely Balady (G1), Chinese (G2), Sids-40 (G3), Egaseed-1 (G4) and Egaseed-2 (G5) were used in the present investigation to study the genetic response to tissue culture technique.

The young leaf parts inside the cloves (foliage leaves) were aseptically isolated and served as explants which used for callus induction, were surface-sterilized by rinsing in 70% ethyl alcohol for 1 minute then washed 3 times with sterile distilled water followed by immersion in 40% of commercial bleach (5.5 w/v sodium hypochloride, NaOC l) for 20 minutes with continuous shaking. Then cloves were washed three times in sterile distilled water under sterile conditions of air laminar flow hood.

Callus induction:

For callus induction, sterilized explants were placed on the surface of 10 ml of sterile callus induction medium in screw capped glass vials (50 x 100 mm). The basic medium used for callus induction was consisted of macro and micro salts according to Murashige and Skoog (1962) supplemented with three concentrations (2mg/L, 4mg/L and 6mg/L) of 2,4- dichlorophenoxy acetic acid (2,4-D). The vials were incubated at 25 ± 2ºC in continual darkness for two weeks and then incubated for additional 4 weeks at 25±2^{o}C under continuous illumination derived from cool white fluorescent tubes.

Plant regeneration:

The sufficiently developed calli were removed from the explants and transferred onto regeneration medium, MS salt medium supplemented with different combinations of Kinetene (Kin) and Indole-3- acetic acid (IAA) (0.1 mg/L IAA + 2 mg/L Kin, 1 mg/L IAA + 1 mg/L Kin and 0.1 mg/L IAA + 4 mg/L Kin). The transferred callus cultures were incubated at 25±2^{o}C in continual darkness for one week and then incubated for additional 4 weeks at 25±2^{o}C under continuous illumination derived from cool white fluorescent tubes. The pH of all media was adjusted to be 5.8 before the addition of 0.8 % (W.V) agar. The media were autoclaved at (121^{o}C for 20 minutes) at 1.2Kg/cm^{2}.

Statistical Analysis:

The response to tissue culture was evaluated in the basis of the following parameters:

- Callus formation (C %), calculated as percentage of explants produced callus from the total cultured explants.
- Percent of shoots, calculated as percentage of explants produced shoots from the total explants produced callus.
- Number of shoots per explant.
- Percent of roots, calculated as percentage of explants produced roots from the total explants produced callus.

A completely randomized block design with three replications (each replicate containing three vials, each vials containing 5 explants) per genotype was used in analysis of the recorded values of % of callus induction, % of shoots, number of shoot per explant and % of root.

Analysis of variance and LSD test were used to examine the significance of differences between genotypes (five parents) and media effect on callus induction, percentage of shoots, number of regenerated shoots and percentage of root. Data analysis was performed by MSTAT.C (ver. 2.10, 1992) computer statistical analysis program.

Cluster analysis of the standardized tissue culture traits was carried out based on the Euclidian distance coefficient and un-weighted pair group method with arithmetic means (UPGMA) using NTSYS-pc version 2.11T (Rolhf, 2000).

Molecular markers:

DNA extraction:

Genomic DNA was isolated from fresh leaves, bulked from 5 different plants per genotype using CTAB protocol for plants (Doyle and Doyle,1987). RNA was removed from the DNA preparation by adding 10 µl of RNAase (10mg/ml) and then

incubated for 30 min at 37°C. DNA sample concentration was quantified by using a spectrophotometer.

ISSR analysis:

Seven ISSR primers (Table 1), obtained from (Metabion International AG, Germany) were tested in the present experiment, to amplify the template DNA. Amplification reactions were carried out in 25µL volumes, containing 3.0 µL 10X PCR buffer (Promega), 4.0 µL of 25 mM Mg Cl_2, 3.0 µL dNTPs (each 2.5 mM) mix (Promega), 0.3 µLTaq DNA polymerase (5units/uL, Promega), 11.0 µL dH2O, 2.0 µL of 10 pmol/µL primer and finally 1 µL DNA (25 ug/uL) template. Amplification conditions were carried out in a thermal Cycler (Model SensoQuest, GmbH, Germany) with the following specification; 5 minutes at 94ºC initial denaturation; followed by 45 cycles of 1 minute at 92ºC, 1 minute based on primer annealing, 2 minutes at 72ºC extension, then 10 minutes at 72ºC.

SRAP analysis:

Nine SRAP primers (Table 1), obtained from (Metabion International AG) were tested in the present experiment, to amplify the template DNA. Amplification reactions were carried out in 25µL volumes, containing 3.0 µL 10X PCR buffer (Promega), 4.0 µLof 25 mM MgCl2, 3.0 µL dNTPs (each 2.5 mM) mix (Promega), 0.3 µLTaq DNA polymerase (5units/uL, Promega), 11.0 µL dH2O, 1.0µL of each of forward and reverse primer (10 pmol/µL primer), and finally 1 µL DNA (25 ug/uL) template. Amplification conditions were carried out in a thermal Cycler (Model SensoQuest, GmbH, Germany) with the following specification; initial 5 minutes at 94°C followed by 10 cycles of 1 minute at 94°C, 1 minute at 35°C and 2 minutes at 72°C, then 35 cycles of 1 minute at 94°C, 1 minute at 50°C, 2 minutes at 72°C, and final 10 minutes extension at 72ºC.

Amplification products were separated by horizontal gel electrophoresis unit using 2% for (ISSR) and 2.5% for (SRAP) agarose gel. Electrophoresis was carried out under constant voltage of around 80V for approximately 3-3.5 hours. The gel was stained with ethidium bromide and visualized using GelDoc-It®2 Imager (www.uvp.com).

Data analyses:

ISSR and SRAP-based molecular markers were scored visually using the software package MVSP (Multi-Variate Statistical Package) and DNA bands were scored as present (1) or absent (0). The pairwise comparisons between the tested isolates were used to calculate the coefficient of genetic similarity matrix (Gs) according to Dice (1945). Cluster analysis was presented as the dendrogram based on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA).

The correlation coefficients between the Euclidean distance matrix based on tissue culture traits and genetic distance matrix obtained with molecular markers were calculated according to Mantel (Mantel, 1967) using NTSYS-pc.

Table1. Primer sequences and codes used.

Primer codes		Sequence (5' to 3')
ISSR	HB05	5'-CAC ACA CAC ACA AC -3'
	HB10	5'-GAG AGA GAG AGA CC -3'
	HB08	5'-GAG AGA GAG AGA GG -3'
	HB14	5'-GTG GTG GTG GC-3'
	HB15	5'-GTG GTG GTG GC-3'
	844A	5'-CTCTCTCTCTCTCTCTAG-3'
	814	5'-CTCTCTCTCTCTCTCTTG-3'
SRAP		
SRAP-1	Em 1a	5′–GAC TGC GTA CGA ATT AAT–3′
	Me1b	5′–TGA GTC CAA ACC GGA AG–3′
SRAP-2	Em 1a	5′–GAC TGC GTA CGA ATT AAT–3′
	Me2	5′–TGA GTC CAA ACC GG AGC–3′
SRAP-3	Em 1a	5′–GAC TGC GTA CGA ATT AAT–3′
	Me3	5′–TGA GTC CAA ACC GGA AT–3′
SRAP-4	Em 1a	5′–GAC TGC GTA CGA ATT AAT–3′
	Me4	5′–TGA GTC CAA ACC GGA CC–3′
SRAP-5	Em 1c	5′–GAC TGC GTA CGA ATT AAC–3′
	Me1b	5′–TGA GTC CAA ACC GGA AG–3′
SRAP-6	Em 1c	5′–GAC TGC GTA CGA ATT AAC–3′
	Me2	5′–TGA GTC CAA ACC GG AGC–3′
SRAP-7	Em 1c	5′–GAC TGC GTA CGA ATT AAC–3′
	Me3	5′–TGA GTC CAA ACC GGA AT–3′
SRAP-8	Em 2	5′–GAC TGC GTA CGA ATT TGC–3′
	Me3	5′–TGA GTC CAA ACC GGA AT–3′
SRAP-9	Em 2	5′–GAC TGC GTA CGA ATT TGC–3′
	Me1b	5′–TGA GTC CAA ACC GGA AG–3′

Results and Discussion

Tissue culture response:

The different stages of in vitro regeneration of different garlic genotypes from cultured explants on callus induction medium to rooted plants formation on regeneration medium are shown in Fig. 1.

The mean percentages for callus formation (C%), shoots (Sh%), roots (Rt%) and the mean number of shoots per explant (No. Sh/exp) for the five garlic genotypes are shown in Table (2).

The results revealed that calli were formed from all tested garlic genotypes. However, the percentage of callus formation varied from one genotype to another according to the concentrations of 2,4-D (Table 2). On average, the percentage of callus formation ranged from 48.88% for G5 to 63.45% for G1. These differences between the tested genotypes were highly significant (Table 3).

High significant differences in C % were also found between the tested levels of 2,4-D (Table-3). Overall Genotypes the results in Table (2) showed that the callus induction medium containing 4 mg/L 2,4-D exhibited the highest percentages of callus formation (65.38%). In addition, the significant interaction between the genotype and concentration of the 2,4-D also revealed that the genotype performed differently from one type concentration to another.

The calli obtained from the tested genotypes which were transferred and sub-culture on regeneration medium supplemented with different combinations of Kin and IAA developed shoots after about 3 – 4

weeks of subculture. The results in Table (2) showed that the genotype G2 possessed the highest percentage of shoots 45.73%, followed by G1 (43.52%) and G3 (42.61%) while the lowest percentage of shoots were obtained by G4 and G5 (37.14% and 35.83%, respectively). The analyses of variance revealed highly significant differences between the five genotypes (Table 3).

Overall genotypes, the regeneration medium containing 0.1 mg/L IAA and 2 mg/L Kin revealed the highest % of shoot formation (60.69 %), while the regeneration medium with 1 mg/L IAA and 1 mg/L Kin was the lowest one (15.15%). These differences between the medium were high significant (Table-3). The analysis of variance also revealed that the interaction between genotype and concentration were highly significant (Table 3). This indicating that the regeneration rate depends on the genotype and concentration of the growth hormones.

The results in Table (2) showed that the genotype G2 possessed the highest number of shoots per explant (7.12 sh/exp), followed by G3 (6.31 sh/exp) and G1 (5.82 sh/exp) while the lowest number of shoots/explant were obtained by G4 and G5 (4.1 and 2.55 shots/explant, respectively).

Overall genotypes, the regeneration medium with 0.1 mg/L IAA and 2 mg/L Kin exhibited the highest shoot/explant (6.88%), while the 1 mg/L IAA and 1 mg/L Kin was the lowest one (2.09%). The analyses of variance revealed highly significant differences between the genotype, the concentration of Kin and IAA as well as the interaction between them (Table 3).

The results in (Table 2) also showed that the G2 followed by G1 and G3 exhibited the highest percentages of root formation (39.03, 33.83 and 33.3%, respectively) while the G5 and G4 exhibited the lowest percentages of root formation (27.01 and 26.81%, respectively). Overall genotypes, the regeneration medium with 0.1 mg/L IAA and 2 mg/L Kin medium revealed the highest % of root formation (42.21%), while the regeneration medium with 1 mg/L IAA and 1 mg/L Kin was the lowest one (23.84%). The analyses of variance revealed highly significant differences between the genotype, the concentration of Kin and IAA as well as the interaction between them (Table 3). Overall genotypes, the genotype G2 followed by G1 and G3 were highly responsive for *in vitro* culture while the two genotypes G4 and G5 were less responsive for *in vitro* culture. These results revealed that callus induction medium supplemented with 4 mg/L 2,4-D was the best medium for callus induction from garlic genotypes while the regeneration medium containing 0.1 mg IAA + 2 mg/L Kin was the best for plant regeneration from garlic genotypes.

These results are similar to those obtained by Barandiarian *et al.* (1999a), who produced 72% induced calluses and less than 30% regenerating calluses for the red-garlic group.

Haque *et al.*(2000) and Fišerová *et al.* (2016) indicates considerable differences in the in vitro cloves formation and multiplication factor for some Japanese varieties of garlic but

without affecting by growth retardants.

Several reports showed the effects of 2,4-D in different garlic cultivars (Myers and Simon 1999; Barandarian *et al.,* 1999b; Robledo-Paz *et al.,* 2000; Sata *et al.*, 2000; Fereol *et al*., 2002).

De Klerk *et al.* (1997) and Guohua (1998) reported that auxins induce callus formation, proliferation and somatic embryogenesis while cytokinins induce mostly shoot and root differentiation and elongation.

Table 2. Mean percentages of culls formation (C%), shoots (Sh%), roots (Rt%) and mean number of shoot/exp (No. Sh/exp) for garlic explants on culture medium supplemented with different concentration of growth regulators.

Traits	Genotypes	Midia			Mean
		2 mg/L 2,4-D	4 mg/L 2,4-D	6 mg/L 2,4-D	
% of callus formation	G1	63.03	72.44	54.89	63.45
	G2	47.54	64.53	44.64	52.24
	G3	63.90	68.97	49.17	60.68
	G4	49.44	61.14	55.21	55.26
	G5	47.49	59.86	39.29	48.88
	Mean	54.28	65.38	48.64	56.10
	LSD Genotypes (G)	0.05%	1.86	0.01%	2.51
	LSD Midia (M)		1.44		1.95
	LSD G*M		3.22		4.35
Traits	**Genotypes**	**Midia**			**Mean**
		0.1 mg/L IAA+ 2 mg/L Kin	**1 mg/L IAA+ 1 mg/L Kin**	**0.1 mg/L IAA+ 4 mg/L Kin**	
% of shoot formation	G1	63.97	17.45	49.13	43.52
	G2	68.46	17.85	50.89	45.73
	G3	63.90	15.81	48.12	42.61
	G4	54.37	13.24	43.82	37.14
	G5	52.73	11.40	43.36	35.83
	Mean	60.69	15.15	47.06	40.97
	LSD Genotypes (G)	0.05%	1.51	0.01%	2.05
	LSD Midia (M)		1.17		1.58
	LSD G*M		2.62		3.54
No. Of shpoots/ Exp	G1	8.50	2.56	6.40	5.82
	G2	11.23	2.90	7.22	7.12
	G3	8.72	2.71	7.50	6.31
	G4	3.10	1.91	7.28	4.10
	G5	2.82	0.38	4.46	2.55
	Mean	6.88	2.09	6.57	5.18
	LSD Genotypes (G)	0.05%	1.02	0.01%	1.37
	LSD Midia (M)		0.79		1.07
	LSD G*M		1.77		2.38
% of root formation	G1	44.83	23.92	32.75	33.83
	G2	53.54	26.79	36.75	39.03
	G3	43.64	24.78	31.48	33.30
	G4	31.92	24.50	24.01	26.81
	G5	37.09	19.22	24.73	27.01
	Mean	42.21	23.84	29.94	32.00
	LSD Genotypes (G)	0.05%	1.51	0.01%	2.05
	LSD Midia (M)		1.17		1.58
	LSD G*M		2.62		3.54

Table 3. The analysis of variance of mean percentage of callus formation (C%), shoots (Sh%), roots (Rt) and the number of shoots/explant (No. Sh/exp) for the five garlic genotypes.

Source of variation	df	Ms			
		%C	% Sh	No.Sh/exp	% Rt
Replicates	2	1.91	11.5	1.251	11.5
Genotypes (G)	4	321.24**	164.09**	30.404**	238.99**
Midia (M)	2	1088.45**	8194.9**	107.601**	1312.29**
G*M	8	57.58**	15.35**	9.61**	37.91**
Error	28	3.72	2.46	1.114	2.46

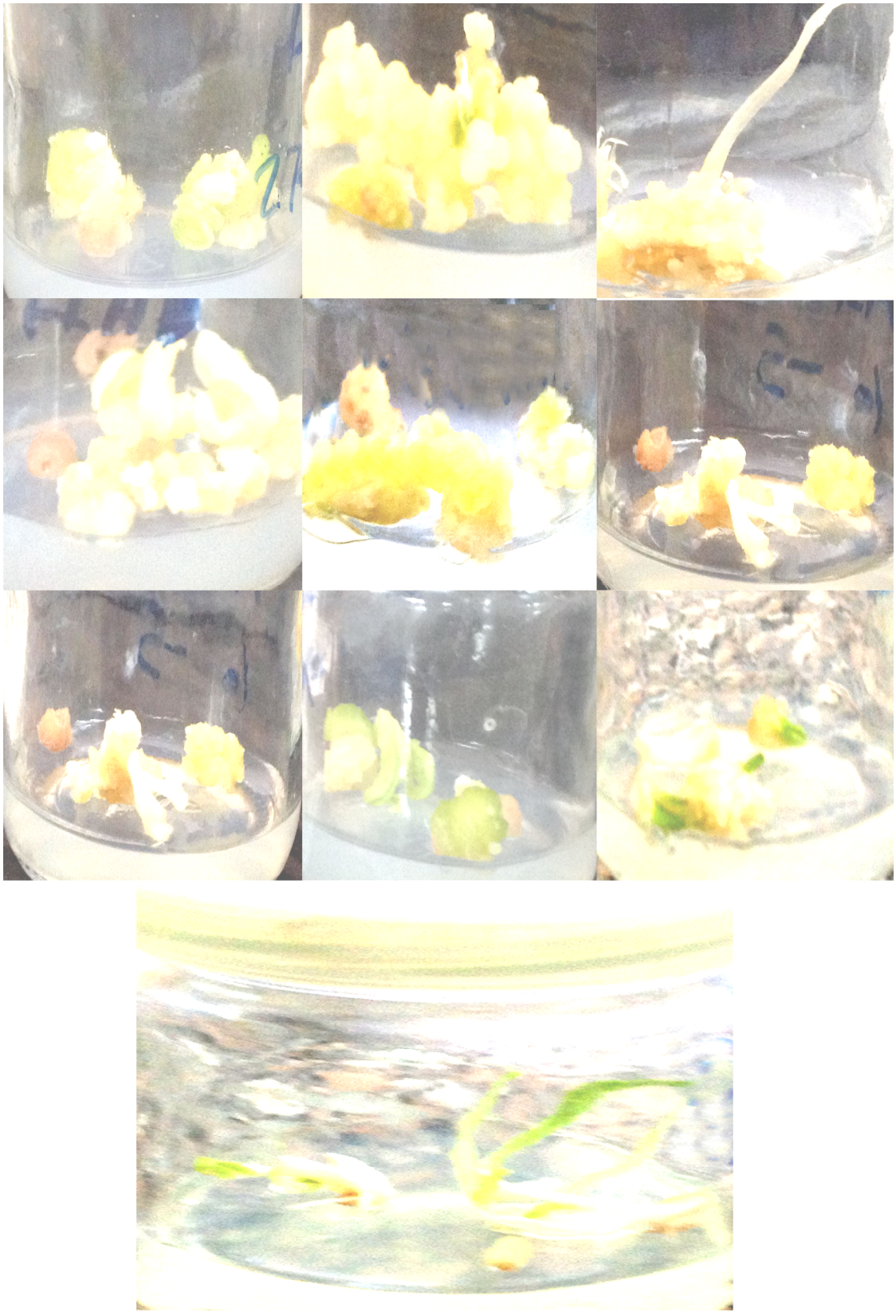

Fig. 1. Explants with developed callus and adventitious bud formation on callus induction medium, Shoots and roots development after 4 weeks on shoot regeneration medium.

Genetic relationships using tissue culture traits:

Euclidean distance

Relationships among the five garlic genotypes based on standardized values of the tissue culture traits are presented in Table (4). The dissimilarity matrix of the Euclidean distance using tissue culture traits between all pairs of genotypes ranged from 0.59 between G1 and G3 to 4.16 between G2 and G5.

Cluster analysis

A dendrogram generated from the standardized tissue culture data is presented in Figure 2. The UPGMA dendrgram separated the five garlic genotypes into two clusters; the first cluster comprised three garlic genotypes (G1, G2 and G3) which were highly responsive for tissue culture. The second cluster comprised the two other garlic genotypes (G4 and G5) which were the less responsive in tissue culture generative ability.

The range of Euclidean distance among all genotypes (0.56 – 4.16) was relatively wide, indicating that the amount of variation in tissue culture traits among the genotypes is relatively high. These values are assumed to reflect the genetic diversity of the loci controlling these traits, indicating the possibility of selecting genotypes that have a diverse genetic background and the prospect of obtaining broad segregation for the traits.

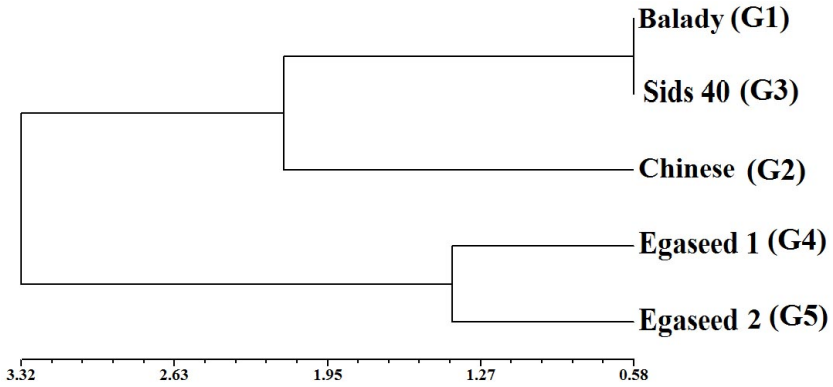

Fig. 2. Dendrogram obtained from UPGMA cluster based on tissue culture data from the five garlic genotypes.

Table 4. **Euclidean Distance matrix of five garlic genotypes using tissue culture traits.**

	G1	G2	G3	G4	G5
G1	00.00				
G2	2.30	00.00			
G3	0.59	1.99	00.00		
G4	2.62	3.55	2.345	00.00	
G5	3.76	4.16	3.477	1.393	00.00

Molecular characterization: Polymorphism and primer evaluation

The seven selected ISSR primers produced a total of 65 bands with size ranging from 147 bp (HB05) to 955 bp (HB08) (Fig. 3). Out of them, 38 (58.46%) bands were polymorphic with an average of 5.43 polymorphic bands per ISSR primer (Table 5). The number of polymorphic bands ranged from 3 for ISSR-814 primer to 8 for HB15 primer. The polymorphic information content (PIC) value was significantly high ranging from 0.13 (ISSR-814) to 0.32 (HB15) with an average value of 0.23 proving the usefulness of ISSR primers in detecting polymorphism across the five garlic genotypes. Further, the nine SRAP primer combinations generated a total of 126 bands, with size ranging from 48 bp (SRAP-8) to 1367 bp (SRAP-7) (Fig. 4). Out of them, 69 (54.91 %) bands were polymorphic. The number of polymorphic bands with SRAP primers ranged from 4 for SRAP-8 to 12 for SRAP-2 with an average of 7.67 (Table 5). A maximum PIC value of 0.31 was observed using SRAP-9 whereas a least PIC value of 0.13 was obtained using SRAP-6 and SRAP-7 primers. The average PIC value obtained using SRAP markers was 0.21.

The resolving power (Rp) of the seven ISSR primers ranged from 2.0 for primers HB08 and ISSR-814 to 4.8 for primer HB15 with a mean of 3.09. Two ISSR primers (HB15 and ISSR-844A) possess the highest Rp values (4.8 and 4.0, respectively) and are each able to distinguish all 5 garlic genotypes (Table 5). Interestingly, higher Rp was observed for SRAP primer combinations, ranging from 2.4 for SRAP-8 to 8.0 for SRAP-2 with a mean Rp of 4.4. Out of the nine SRAP primer combinations, two combinations (SRAP-2, and SRAP-9) possess the highest Rp values (8.0, and 6.4, respectively) and also have the ability to distinguish all 5 garlic genotypes (Table 5).

Unique DNA fragments with different sizes were detected in particular genotype but not in the others using different primers. The presence of a unique band for a given genotype is referred as positive marker while the absence of a common band served as negative marker. Such bands could be used as DNA markers for genotype identification and discrimination (Table 6). In this respect, the genotype G2 possessed the highest number of unique bands (7 positive DNA bands and 23 negative DNA bands) while the genotype G1 exhibited the lowest number of unique bands (3 positive DNA bands and 2 negative DNA bands) (Table 6).

For two molecular markers system, ISSR and SRAP primers, a total of 191 DNA fragments were amplified by 16 primers from all genotypes with an average 11.94 bands/primers. Out of fragments, 107 (56.02 %) showed polymorphism and 84 (43.98 %) were common bands. The results showed that the genotype G3 displayed the highest number of DNA fragments (162 bands), while the genotype Egaseed 2 (G5) revealed the least number of bands (130 bands).

Similar findings were earlier reported by Jabbes *et al.* (2011) screened as many as 35 garlic genotypes using 7 ISSR markers, Shaaf *et al.* (2014) evaluated 31 garlic geno-

types using 6 ISSR markers, Chen *et al.* (2014) screened 39 genotypes using ISSR primers and Chen *et al.* (2013) evaluated 40 garlic germplasms using 23 sequence-related amplified polymorphism (SRAP) primer combinations.

Genetic diversity

The genetic similarity based on ISSR, SRAP and its combined data among five garlic genotypes are shown in Table 7. Based on the ISSR markers data, the genetic similarity ranged from 0.703 between G2 and G4 to 0.875 between G3 and G4. The genetic similarity based on SRAP primer combinations data ranged from 0.712 between G2 and G4 to 0.966 between G4 and G5. Therefore, in order to explain the genetic diversity and gene differentiation precisely, the subsequent analyses were carried out with ISSR + SRAP data. The combination of two marker types resulted in percentage polymorphic bands (PPB) of 56.61 % in all the five garlic genotypes investigated. The minimum genetic similarity of 0.709 was observed between G2 and G4. In contrast, the maximum genetic similarity of 0.932 was found between G4 and G5. The high level of polymorphism and large range of genetic distance from 0.068 to 0.291 indicates the existence of high relatively genetic diversity among investigated garlic genotypes.

Cluster analysis

UPGMA clustering of the 5 genotypes based on the Dice similarity was shown in Fig. 5 a–c. Based on similarity matrix of ISSR, a dendrogram (Fig. 5a) separated the five genotypes into two major clusters. Cluster I comprised of G1 and G2 while cluster II contained G3, G4 and G5. The results of SRAP markers (Fig. 5b) also supported that all genotypes were divided into two major clusters (I, II). However, unlike with ISSR analysis, the genotype G2 was separated in a single branch from the other genotypes and comprised cluster I. The cluster II was composed of four genotypes G1, G3, G4 and G5, forming two sub-clusters. Sub-cluster I comprised of G1 and G3. Both of them appeared to be more similar with a 0.889 similarity coefficient. The genotype G4 and G5 formed the sub-cluster II with genetic similarity 0.966.

The Dice's genetic similarity matrix based on combined ISSR and SRAP data was used to generated a dendrogram showing genetic relationships among the genotypes. The dendrogram grouped the five genotypes into two main clusters (Fig. 5c). Same to the SRAP analysis, the genotype G2 was separated in a single branch from the other genotypes with genetic similarity 0.758 and comprised cluster I. The four genotypes G1, G3, G4 and G5 formed cluster II with 0.827 GS. The cluster II separated into 2 sub-clusters (I a, and I b). The G1 and G3 genotypes occupied the sub-cluster I with 0.873 GS, whereas the genotypes G4 and G5 were placed in sub-cluster II with 0.932 GS.

In order to compare the extent of agreement between dendrograms derived from two molecular marker systems, a distance matrix was constructed for each assay and compared using Mantel matrix correspondence test. Significant correlation was found

between ISSR and SRAP markers (r = 0.751: p = 0.001).

The high level of polymorphism detected in the present study using ISSR and SRAP analysis and the determination of DNA specific markers for the garlic genotypes, suggested that these approaches showed considerable potential for garlic genotype identification and discrimination

The high level of genetic variation observed in this study is consistent with the results from previous studies of garlic carried out using different molecular markers (Ipek *et al.,* 2003, 2005 and 2008; Lampasona *et al.,* 2003; Volk *et al.,* 2004, chen *et al.,* 2013; Rakesh *et al.,* 2018), thereby confirming the great diversity among garlic accessions.

Correlation dissimilarity matrix between tissue culture traits and molecular markers:

The patterns obtained with ISSR primers, HB05 (416 bp), HB10 (286 bp) and HB14 (292 bp), further the patterns obtained with SRAP primer combinations, SRAP-1 (209 bp), SRAP-2 (753 bp), SRAP-4 (363 and 170 bp), SRAP-5 (177 bp), SRAP-6 (565 bp), SRAP-7 (545 bp) and SRAP-9 (677 bp, 614 bp and 416 bp) primer combinations for genotypes (G1, G2 and G3) suggest that these primers or primer combinations have the ability to produce molecular markers for tissue culture response in garlic cultivars. Since these fragments were visualized using these primer or primer combinations in the genomic DNA of highly responsive tissue culture genotypes while were absent in the low responsive genotypes (G4 and G5), these fragments can be considered as positive markers for tissue culture response in garlic (Fig. 3 and 4).

In order to compare the extent of agreement between dendrograms derived from tissue culture traits and two molecular markers (ISSR and SRAP), a distance matrix was constructed for each assay and compared using Mantel matrix correspondence test. Significant correlation was found between ISSR and SRAP markers (r = 0.751: p = 0.001). A highly significant positive correlations were found between ISSR marker and tissue culture traits and between SRAP marker and tissue culture traits (r = 0.642 and r = 0.526: p = 0.001, respectively), as well as highly significant positive correlation was found between combined ISSR + SRAP markers and tissue culture traits (r = 0.595: p = 0.001).

The significant correlation indicate that these independent sets of data likely reflect the same pattern of genetic diversity and validate the use of these data to calculate the different diversity statistics for tissue culture response in the peanut genotypes.

Similar conclusion was obtained in different garlic genotypes by Ipek *et al.,* 2008; Lampasona *et al.,* 2003; Volk *et al.,* 2004, chen *et al.,* 2013.

Table 5. Parameters of genetic diversity.

Primers	TB	PB	%PB	PIC	MI	RP
HB05	8.00	4.00	50.00	0.20	0.80	2.40
HB10	9.00	6.00	66.67	0.23	1.39	2.80
HB08	6.00	4.00	66.67	0.24	0.96	2.00
HB14	11.00	6.00	54.55	0.22	1.31	3.60
HB15	10.00	8.00	80.00	0.32	2.56	4.80
844A	11.00	7.00	63.64	0.25	1.73	4.00
814	10.00	3.00	30.00	0.13	0.38	2.00
SUM	65.00	38.00	----	1.58	9.13	21.60
AVR	9.29	5.43	58.79	0.23	1.30	3.09
SRAP-1	17.00	9.00	52.94	0.21	1.86	5.20
SRAP-2	19.00	12.00	63.16	0.27	3.23	8.00
SRAP-3	11.00	7.00	63.64	0.22	1.53	3.20
SRAP-4	11.00	7.00	63.64	0.25	1.73	4.00
SRAP-5	13.00	7.00	53.85	0.21	1.46	4.00
SRAP-6	13.00	7.00	53.85	0.18	1.29	3.20
SRAP-7	16.00	5.00	31.25	0.13	0.65	3.20
SRAP-8	12.00	4.00	33.33	0.13	0.53	2.40
SRAP-9	14.00	11.00	78.57	0.31	3.39	6.40
SUM	126.0	69.00	494.22	1.91	15.69	39.60
AVR	14.00	7.67	54.91	0.21	1.74	4.40
SUM	191.00	107.00	----	3.49	24.82	61.20
AVR	11.94	6.69	56.61	0.22	1.55	3.83

TB=Total Bands; PB=Polymorphic bands; PPB=%Polymorphic bands; PIC=polymorphism information content; MI=Marker index; RP=Resolving power

Table 6. Positive and negative unique DNA bands.

Sr. no	Genotypes	Markers	
ISSR marker		Unique positive	Unique negative
1	G1	387 bp (814)	469 bp (HB10)
2	G2	435 bp (HB08)	409 bp (HB05)
		275 bp (844A)	623, 393 bp (HB10
		------	359, 221 bp (HB15)
3	G3	241 bp (HB10)	------
		280, 239 bp (HB15)	------
		428 bp (844A)	------
4	G4	377 bp (HB14)	382 bp (HB08)
		------	451 bp (HB14)
		------	450 bp (844A)
5	G5	955 bp (HB08)	173 bp (HB05)
		------	324 bp (HB10)
		------	482 bp (HB14)
		------	892 bp (844A)
SRAP			
1	G1	191 bp (SRAP-4)	637 bp (SRAP-2)
		592 bp (SRAP-7)	------
2	G2	297 bp (SRAP-1)	635 bp (SRAP-1)
		308 bp (SRAP-3)	499 bp (SRAP-2)
		422 (SRAP-8)	666, 493, 453, 291 bp (SRAP-3)
		502, 290 bp (SRAP-9)	1022, 493 bp (SRAP-4)
		------	318, 271, 151 bp (SRAP-5)
		------	649, 376, 334, 183 (SRAP-6)
		------	205 bp (SRAP-9)
3	G3	375, 270 bp (SRAP-1)	187 bp (SRAP-1)
		321 bp (SRAP-2)	470 bp (SRAP-5)
		161 bp SRAP-6)	------
		353, 277 (SRAP-9)	------
4	G4	322 bp (SRAP-4)	402 bp (SRAP-2)
		194 bp (SRAP-7)	
5	G5	------	117 bp (SRAP-6)
		------	369 bp (SRAP-8)

Table 7. Genetic similarity values calculated from DNA fragments generated with 7 ISSR, 9 SRAP primer or primer combinations and combined data of them in six peanut genotypes.

ISSR	G1	G2	G3	G4	G5
G1	1				
G2	0.863	1			
G3	0.84	0.784	1		
G4	0.787	0.703	0.875	1	
G5	0.804	0.719	0.787	0.864	1
SRAP	G1	G2	G3	G4	G5
G1	1				
G2	0.791	1			
G3	0.889	0.792	1		
G4	0.834	0.712	0.832	1	
G5	0.842	0.717	0.828	0.966	1
Combined	G1	G2	G3	G4	G5
G1	1				
G2	0.816	1			
G3	0.873	0.789	1		
G4	0.819	0.709	0.846	1	
G5	0.829	0.718	0.815	0.932	1

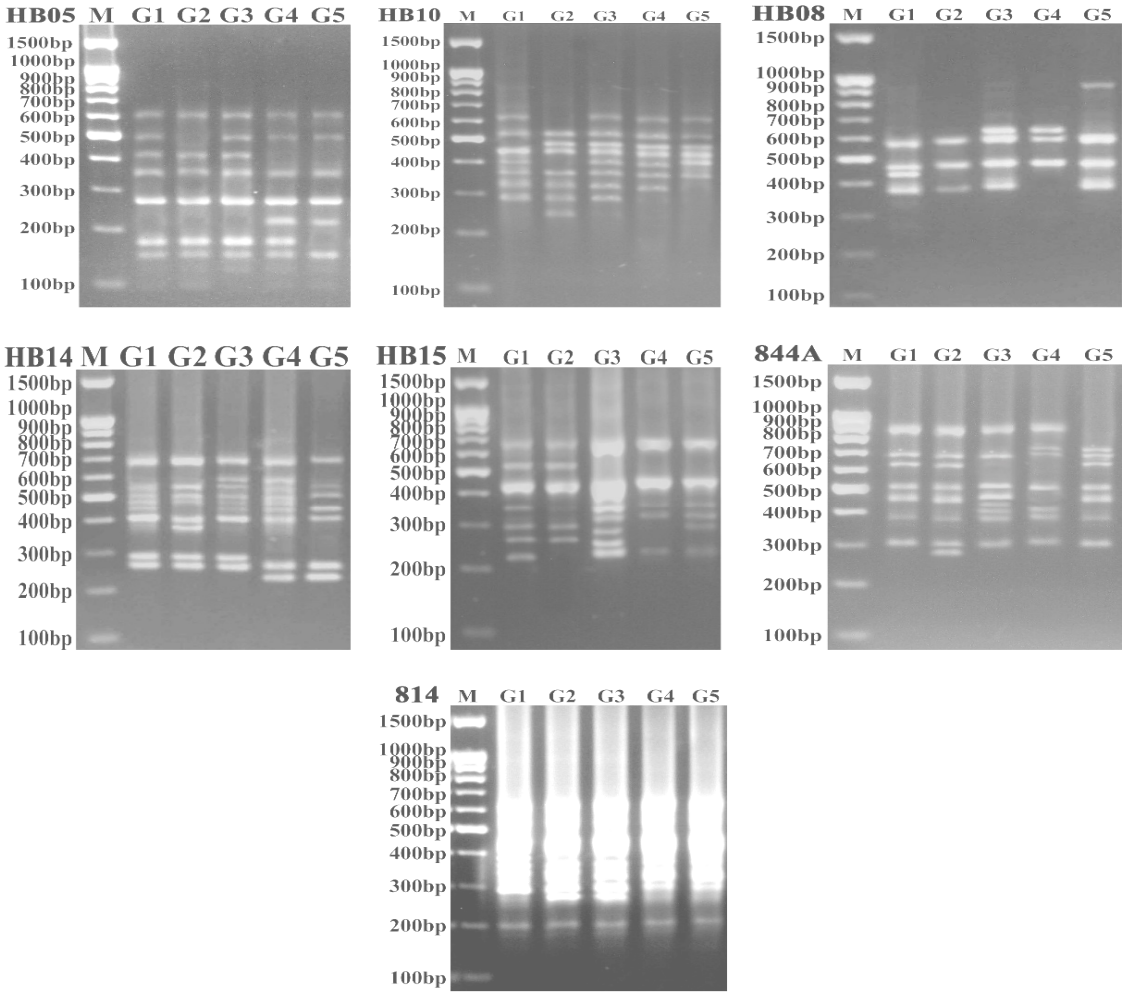

Fig. (3): Agarose gel electrophoresis of ISSR profile in five Garlic genotypes.

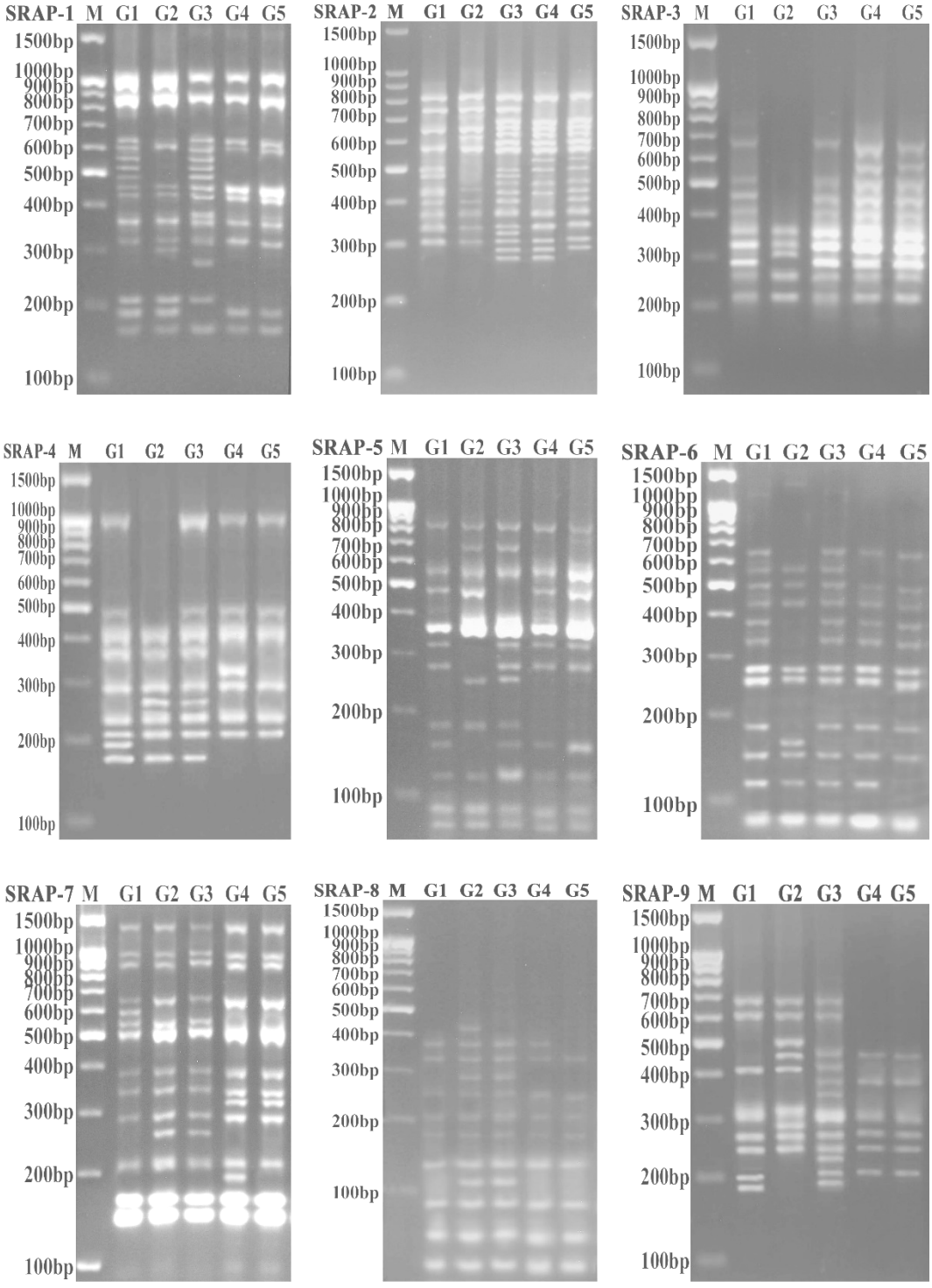

Fig. (4): Agarose gel electrophoresis of SRAP profile in five Garlic genotypes.

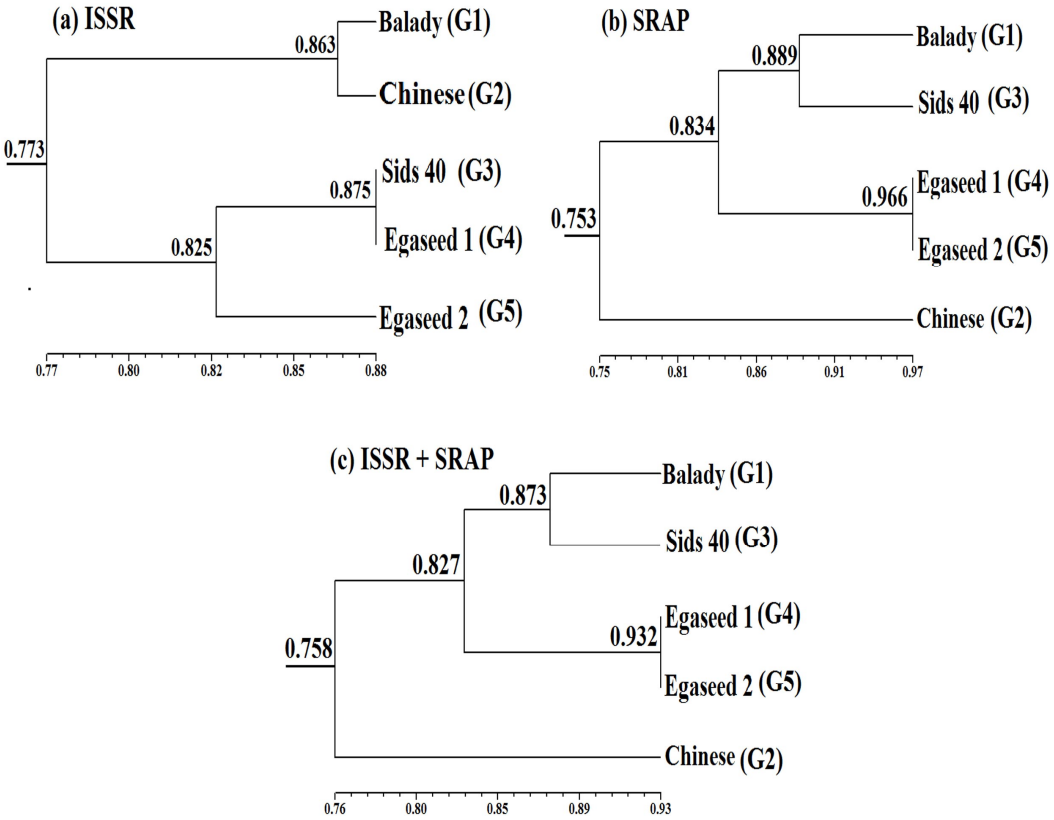

Fig. (5 a-c): Dendrogram of five garlic genotypes developed from (a) ISSR (b) SRAP and (c) combined ISSR and SRAP data using UPGMA analysis. The scale is based on Dice coefficients of similarity.

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الواسمات الجزيئية الوراثية والاستجابة لزراعة الأنسجة في الثوم

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الملخص

استخدمت في هذه الدراسة خمسة أصناف من الثوم هي (بلدي و صيني و سدس ٤٠ وإيجاسيد ١ و إيجاسيد ٢) وذلك لدراسة الاستجابة لزراعة الأنسجة والاختلافات الوراثية بين هذه الأصناف كما تم استخدام الواسمات الجزيئية التالية (الـ ISSR, SRAP) لتحديد الواسمات المرتبطة بالاستجابة لزراعة الأنسجة. وأظهر تحليل التباين اختلافات معنوية بين الأصناف والتركيزات المختلفة لمنظمات النمو والتفاعل بينهما لجميع صفات زراعة الأنسجة. وأوضح التحليل العنقودي لبيانات زراعة الأنسجة انعزال الأصناف في مجموعتين تضم المجموعة الأولى الأصناف الثلاثة (بلدي وصيني وسدس ٤٠) عالية الاستجابة لزراعة الأنسجة، بينما تضمنت المجموعة الثانية الأصناف منخفضة الاستجابة (إيجاسيد ١ وإيجاسيد ٢). وباستخدام الواسمات الجزيئية تم الحصول على ١٩١ حزمة منهم ١٠٧ حزمة (٥٦,٦١%) أظهرت أشكالاً متعددة. وأظهر التحليل العنقودي لبيانات الواسمات الجزيئية انعزال الأصناف في مجموعتين تضم المجموعة الأولى الصنف الصيني عالي الاستجابة لزراعة الأنسجة بينما تضمنت المجموعة الثانية الأصناف الأقل استجابة لمزارع الأنسجة. كما أظهرت النتائج وجود ارتباط معنوي موجب ($r = 0.595$: $p = 0.001$) بين الواسمات الجزيئية وصفات الاستجابة لزراعة الأنسجة. وأظهرت النتائج وجود ثلاثة عشرة حزمة يمكن استخدامها كواسمات جزيئية للاستجابة لزراعة الأنسجة في أصناف الثوم المستخدمة في هذه الدراسة.

وقد أوضحت النتائج أن الواسمات الجزيئية التى تم استخدامها كانت مفيدة في دراسة وتحديد الاختلافات الوراثية بين هذه الأصناف وأيضاً في تحديد واسمات للاستجابة لزراعة الأنسجة.