Evaluation Of Ten Alfalfa Populations For Forage Yield, Protein Content, Susceptibility To Seedling Damping-off Disease And Associated Biochemical Markers With Levels Of Resistance

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Abstract: Greenhouse and field experiments were carried out at Giza research station to evaluate ten alfalfa genotypes against three fungi causing seedling damping-off disease *(Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani***).** Examined genotypes included two exotic varieties (Cuf-101 and Salt America), seven local populations (Balady, Fixed-N, New valley-1, New valley-2, Siwa, Esmaelia-1 and Esmaelia-94) and New salt population (Sinai 1). *R. solani* was the most causal pathogen reducing significantly fresh and dry shoot yield followed by *M. phaseolina* and *F. oxysporum*, respectively. The local populations Siwa and Ismaelia-1 expressed the best performance for yield superiority and agronomical traits in comparison with the exotic populations. New Salt pop., Salt America and Siwa populations ranked as the highly resistant against seedlings damping-off disease. Selection between and within alfalfa populations for high yielding ability and resistance degree to seedling damping-off disease produce a promising population can be used in the future breeding program. SDS-protein banding patterns of the ten alfalfa populations grown under normal (non-stressed) and fungal stress conditions were found to be useful in developing biochemical markers associated with resistance to damping-off pathogens. The obtained results revealed unique fingerprint characterized for each studied population under non-stress conditions. Similarity indices and consensus tree were developed on the basis of the protein banding patterns of the ten alfalfa populations using protein banding patterns under non-stress conditions. Consensus tree was developed on the basis of the bulked protein banding patterns of the ten alfalfa populations grown in infested soil with the three tested fungi caused seedling damping-off disease. The Dendrogram was gathered the resistant populations in one main cluster and almost all highly susceptible populations together in the same group.

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1. Introduction

Alfalfa (*Medicago sativa* L.) is the largest cultivated forage crop in the world especially in Mediterranean countries. In Egypt, the crop cultivated mainly in North West coast, Ismaelia and New valley governorates. Alfalfa enhances the yield and quality of the following crops by atmospheric nitrogen fixation (Bruulsema and Christie, 1987). Furthermore, alfalfa improves and protects the soil as a result of its robust and perennial root system, fast growing protective canopy and ability to fix atmospheric nitrogen (Shahriari *et al* 2007). It is deep and extensive root system reduces erosion by holding soil together, improves water infiltration and contributes to a rhizosphere conducive to growth of beneficial microorganisms (Rezaee *et al* 2007). It has the highest yield potential and one of the highest feeding values of all adapted perennial forage legumes. Thus, it can be used successfully in many types of livestock feeding programs as pasture, hay, silage, green chop and as a cash crop. Rammah and Hamza (1980) using frequent harvesting method to produce high-quality alfalfa populations. Oushy *et al* (1999) identified genetic variation for the quality and morphological traits, with superior performance of Ismaelia-1 and Ismaelia-94 genotypes. Avci *et al* (2010) recorded significant differences among alfalfa lines and cultivars in dry matter yield and quality traits. Variability for quality components within alfalfa populations has been reported by Sumberg *et al* (1983), Torricelli *et al* (2001) and Julier *et al* (2010).

The persistence and productivity in alfalfa are believed to be influenced by root morphology (Johnson *et al* 1998 and Lamb *et al* 2000b) Snapp *et al* (2003) recorded that the root vigor, the number of lateral root and the root diameter were positively correlated with genotype tolerance to *Fusarium root rot* in snap bean.

Alfalfa plants are subjected to be attack by several soil borne fungal diseases which affecting

survival healthy plants causing subsequently considerable yield loss (Morsy *et al* 2011). The causal pathogenic fungi of damping off and root rot/wilt disease complex are *Rhizoctonia solani, Fusarium oxysporum, Fusarium solani, Verticillium spp. and Macrophomina phaseolina* (Omar and Rammah, 1992, Ellanskaya *et al* 1995, Ismail, 1995, El-Morsy and Belal, 1997 and Morsy *et al* 2011). Several methods of controlling alfalfa root rot/wilt diseases were reported and including screening for disease resistance (Omar and Rammah, 1992), biological control (Abdul-Rahman and Alkhail, 2004) and use of abiotic and biotic agents (Morsy *et al* 2011). Consequences of the incidence of such diseases have significant economical impact, exhibited through thinning of the crops, decrease in yield, reduce quality of forage plants and longevity of alfalfa (Krnjaja, 2005).

Biochemical genetic markers offer specific advantage in assessment of genetic diversity and trait-specific crop improvement. Such markers can facilitate appropriate choice of parents for crosses to mapping/tagging of gene blocks associated to economically important traits and in turn permits marker-assisted selection (MAS) in backcross, pedigree and population improvement programs (Mohan *et al* 1997).

El -Menshawi *et al* (2003) reported that SDS-PAGE protein banding patterns for water soluble fraction of seed storage proteins was successful in generating biochemical genetic markers related to salt tolerance in sorghum. Four bands with the molecular weights of 72.64, 59.59, 46.37 and 22.75 KDa were absent under salt stress conditions in most of the hybrids and could be considered as negative markers; while, a single band of 37.19 KDa was found to be expressed only under salt stress conditions and could be considered as a positive marker.

Khalifa *et al* (2006) reported that successfully generated some new bands considered as positive marker for resistance against damping-off and root-rot diseases and the higher productivity in peanut, while other bands considered positive markers for susceptibility against damping-off and root-rot diseases and the lower productivity in peanut.

Azzam *et al* (2007) developed biochemical markers associated with levels of resistance to *Cowpea Aphid Borne Mosaic Potyvirus* (CABMV) in sesame. They reported that protein band with molecular weight of 82.0 and 38.0 KDa was found as proteins associated with mild to CBAMV symptoms as in Toshky 1 population that irradiated with 250 Gy.

The objectives of the present work are to: a) evaluate the behavior of ten alfalfa populations under non biotic stress (control) and artificial infestation for seedling damping-off pathogens and their forage, dry yield, agronomical traits and protein content%, b) select more tolerant genotypes for seedling damping-off pathogens, c) determinate specific biochemical changes in resistant and susceptible populations under experiment conditions and d) find out biochemical genetic markers for levels of resistance to damping-off pathogens and using protein markers to identify, characterize and establish relationships of the ten studied alfalfa populations.

2. Materials and Methods

Agronomical characters:

Ten alfalfa populations were examined against each of the three fungi causing seedling damping-off disease *(Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani*). These populations included two exotic varieties (Cuf-101 and Salt America), seven local populations (Balady, Fixed-N, New valley-1, New valley-2, Siwa, Esmaelia-1 and Esmaelia-94) and New Salt population, (Sinai 1), which is more adapted to saline soils in North Sinai conditions (Abd El-Naby *et al* 2013). The ten alfalfa populations were planted in infested pots (30 cm x 50 cm) with perforated bottoms to allow drainage and filled with clay and sand (1:1) under greenhouse conditions. The pots irrigated daily with tap water until seedlings emerged. After all the seeds had germinated watering was carried out by immersing into a complete nutrient solution according to standardize doses. Plants were transplanted to the field after the first cut. The entries were laid out in a randomized block design with three replications. Three cuts were taken for selected plants in the field across populations. The agronomical characters were studied for [fresh, dry yield, plant height (cm), stem diameter (cm), tillers plant^{-1}, crown region (cm) and crown diameter (cm) as well as root to shoot ratio %] per each cut.

Protein content was measured at the third harvest for chemical determinations, 200 g dry plant samples per population, across the three fungal pathogens. The dried samples were fine powdered and wet digested according to Chapman and Pratt (1961). Crude protein % (CP) was determined by standard methods (A.O.A.C. 1990) and estimated by multiplying total N by 6.25 (Anonymous 1995).

Pathological studies

Source of fungal isolates

The fungal isolates (*Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani*) were perivously isolated from root roted alfalfa plants collected from Giza research station, Agriculture Research Center (ARC). The fungi were purified and identified according to Barnett (1960) and proved their pathogenicity ability at Legume and Forage disease Res. Dept., Plant Pathology Research Inst., ARC.

Preparation of fungal inoculum

Bottles containing cornmeal-sand medium (3:1w/w) were autoclaved at 121ºC for 30 min. The sterilized bottles were then inoculated with discs (5 mm in diameter) of seven- days old cultures of *Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani* individually. The bottles were then incubated at 25 ± 2ºC for 15 days.

Soil infestation

Fungal inculum of each fungus was mixed with the potted sterilized soil at the rate of 5, 5 and 3% (W/W) for *Fusarium oxysporum, Macrophomina phaseolina and*

Rhizoctonia solani, respectively. The infested soils were watered daily for one week to enhance growth and distribution of the fungal inoculum. The pots containing infested soils were sown with ten alfalfa population seeds. Three replications were used and each replicate represented by a plot. The seeds were sown at the rate of 30 seeds per plot. Control treatment was sown in uninfested soil for each alfalfa population.

Disease assessment

The germinated seeds of the ten tested populations were examined periodically and percentage of (pre-and post-emergence) damping-off disease was recorded after 45 days from sowing. Disease estimation was calculated based on number of seeds that were sown per each pot. Reaction of the tested populations to fungal infection was recorded for each of the three fungi causing seedling damping-off disease individually according to the following scale:

Resistant (R)	≤ 25% reduction in the survival plants
Moderate susceptible (MS)	26-35% reduction in the survival plants
Susceptible (S)	36-45% reduction in the survival plants
Highly susceptible (HS)	≥ 45% reduction in the survival plants

Statistical analysis

Data were analyzed for statistical significance using the SAS (SAS Institute, Inc., Cary, NC). ANOVA and Duncan's Multiple Range tests were used to statistically analyze the data (Duncan 1955).

Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

For direct visual protein comparisons, proteins extracted from leaves of 10 tested alfalfa populations of 30 days old seedlings grown under stress of three fungi causing seedling damping-off disease *(Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani***)** in green house and in pathogen free potted soil (control) then size fractionated based on the molecular weight by SDS-PAGE performed as described by Laemmli (1970). Vertical slab gels (0.75 mm-thick) were cast and electrophoresed using the Bio Rad Mini-Protean II system. Gels were stained with commassie brilliant blue R-250 solution, photographed and scored using gel documentation system manufactured by Alpha Ease FC (Alphaimager 2200), U.S.A. The similarity matrix of the seven commercial local (Ismaelia -1, Ismaelia -94, New valley-1, New valley-2, Fixed N, Balady and Siwa), two exotic population; (Cuf-101 and Salt America) and new promised population (New Salt pop.) were done using Gel works 1D advanced software UVP-England Program. The relationships among them on both the control conditions (non-fungal stress condition) and under the fungal stress conditions as combined analysis revealed by Dendrograms were done using SPSS windows (Version 14) program.

3. Results and Discussion

Reaction of alfalfa populations

Reaction of alfalfa populations to seedling damping-off disease is summarized in Table (1). Differences in susceptibility of the tested alfalfa populations to fungal infection were noted.

Table 1. Reaction of tested alfalfa populations against seedling damping-off disease caused by *Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani* under greenhouse conditions.

Populations	Seedling damping-off %						Mean	
	F. oxysporum	*RT.	*M. phaseolina*	*RT.	*R. solani*	*RT.		
Cuf-101	9.33 e	**R**	29.33 d	**MS**	42.67 bc	**S**	**27.11**	**MS**
Salt America	16.05 e	**R**	1.23 f	**R**	43.21 bc	**S**	**20.16**	**R**
Balady	57.15 b	**HS**	89.29 a	**HS**	75.00 a	**HS**	**73.81**	**HS**
Fixed N	56.00 b	**HS**	52.00 c	**HS**	73.33 a	**HS**	**60.66**	**HS**
New valley 1	80.00 a	**HS**	50.26 c	**HS**	87.69 a	**HS**	**72.65**	**HS**
New Valley 2	61.54 b	**HS**	91.20 a	**HS**	55.13 b	**HS**	**69.29**	**HS**
Siwa	25.72d	**MS**	29.05 d	**MS**	36.19 d	**S**	**30.32**	**MS**
Ismaelia 94	44.00 bc	**S**	72.00 b	**HS**	68.00 ab	**HS**	**61.33**	**HS**
Ismaelia 1	34.62 c	**MS**	32.06 d	**MS**	64.62 ab	**HS**	**43.77**	**S**
New Salt pop	10.03 e	**R**	13.34 e	**R**	39.29 cd	**S**	**20.89**	**R**
Mean	**39.44**		**45.98**		**58.51**		**48.00**	

***RT: Reaction type according to the mentioned scale.**

R: resistant, S: susceptible, MS: moderate susceptible and HS: highly susceptible.

R. solani was the highest percentage of reduction in survival plants followed by *M. phaseolina* and *F. oxysporum*, respectively. Balady, New valley-1, New valley-2, Ismaelia-94 and Fixed-N alfalfa populations ranked as highly susceptible ones, according to fungal infection mentioned scale, judged by the highest percentage of seedling damping-off disease across the three tested fungi. Whereas, Salt America and New salt pop. populations were ranked as resistant ones judged by the lowest percentage of seedling damping-off disease caused by the tested fungi. Other alfalfa populations (Cuf-101 and Siwa) were grouped as moderate susceptible ones.

Alfalfa response against soil-borne fungi was also detected by several investigators (Omar and Rammah, 1992, Yuxia *et al* 2009, Gaige *et al* 2012, Viands *et al* 2012 and Anderson *et al* 2013). All investigators came to conclusion that alfalfa cultivars and lines were differed in their reaction to the tested fungi.

Effect of fungal infection on fresh and dry yield

Effect of seedling damping-off disease on fresh and dry yield over three cuts is presented in (Table 2). Alfalfa populations infected with seedling damping-off fungi (*F. oxysporum, M. phaseolina and R. solani*) had significant effect on percentage reduction of total fresh and dry yield, compared to control treatment.

High significant differences ($P \leq 0.01$) for fresh and dry yield were recorded among alfalfa populations. The mean average of total fresh and dry yield g plant^{-1} of control populations recorded 506.66 and 98.93 g plant^{-1} over populations. New Salt population recorded the highest fresh and dry yield (672.0 and 139.44 g plant^{-1}) followed by Siwa population (658.0 and 137.59 g plant^{-1}), while Balady population was the lowest total Fresh and dry yield (358.33 and 66.38 g) across all populations. The New Salt pop. and the local population Siwa expressed the best performance for yield superiority in comparison with the exotic populations under Giza field conditions. (Table 3)

It was found that *R. solani* had the highest reduction of fresh yield followed by *M. phaseolina* and *F. oxysporum* respectively. Moreover, the reduction was varied among alfalfa populations. New Salt pop., Salt America, Ismaelia-1 and Siwa populations ranked as the best performances against the tested causal pathogens. On the other side, New valley-1 and Balady were the highest affected alfalfa populations. The lowest reductions in fresh yield across the three tested pathogens were (11.67, 12.59 and 12.59%) of New salt pop., salt America and Ismaelia-1 populations, respectively. Cuf-101 was the lowest reduction in dry yield followed by Salt pop. and Salt America population. *Rhizoctonia solani* infection recorded the largest reduction in fresh and dry yield with mean average of 21.11 and 18.74% respectively. Dry yield recorded less reduction % than forage yield per population across the three fungal damping-off disease. Our study found significant differences among populations for fresh and dry yield under pathogenic fungi stress. These results agreed with Blazhev, (1989), Hwang (1992) and Ellanskaya *et al* (1995). The result obtained in terms of dry yield was similar to that of fresh yield reduction with minor differences in the rank within alfalfa populations.

Table 2. Reduction (%) of total forage and dry shoot yield across three cuts of ten alfalfa populations infected with the three pathogenic fungi comparing with control treatment.

Populations	Control g	Fresh yield %				Control g	Dry yield %			
		F. oxysporum	*M. phaseolina*	*R. solani*	Mean		*F. oxysporum*	*M. phaseolina*	*R. solani*	Mean
Cuf-101	508.67 b	7.51 g	17.39 d	16.91 h	**13.94**	110.38 b	1.80 g	5.81 h	15.67 g	**7.76**
Salt America	465.33 c	10.28 d	12.10 h	15.40 i	**12.59**	95.21 b	3.15 f	7.23 f	18.35 e	**9.58**
Balady	385.33 d	18.22 b	21.67 b	27.09 b	**22.33**	66.38 d	10.78 a	12.74 b	23.13 b	**15.55**
Fixed N	456.00 c	12.91 c	18.63 c	22.29 d	**17.94**	81.22 c	7.77 c	9.11 c	18.73 d	**11.87**
New valley 1	452.00 c	20.02 a	23.50 a	29.00 a	**24.17**	77.29 c	9.00 b	13.82 a	20.71 c	**14.51**
New Valley 2	449.33 c	11.51 c	18.63 c	26.89 c	**19.01**	79.53 c	9.09 b	9.11 c	24.89 a	**14.36**
Siwa	658.00 a	9.50 e	12.62 g	18.11 g	**13.41**	137.59 a	4.96 e	7.27 f	14.94 i	**9.06**
Ismaelia 94	458.67 c	8.33 f	16.39 e	19.15 e	**14.62**	94.47 bc	6.05 d	8.75 d	17.60 f	**10.80**
Ismaelia 1	561.33 b	5.27 h	13.63 f	18.88 f	**12.59**	104.41 b	6.00 d	7.45 e	18.37 e	**10.61**
New Salt pop	672.00 a	4.68 i	12.97 g	17.37 g	**11.67**	139.44 a	5.93 d	7.19 g	14.99 h	**9.37**
Mean	**506.66**	**10.82**	**16.75**	**21.11**	**16.23**	**98.93**	**6.45**	**8.85**	**18.74**	**11.35**

Means followed by the same letter are not significantly different at the $P \leq 0.05$.

Performance of shoot and root characters of selected plants across the three pathogen infection

Shoot and root characters of selected plants of the ten alfalfa populations across the three pathogens are show in Table (3). Populations indicated significant differences ($p \leq 0.05$) across traits. Plant height mean ranged from 52.67 cm of New valley-1 population to 63.33 cm of Siwa population. Means of stem diameter varied from 0.35 cm of Balady population to 0.53 of Cuf-101 population. Also, number of tillers ranged from 13.67 to 23.03 of Balady and Siwa populations, respectively. The best percentages of root/shoot ratio, with minor differences, were recorded of Cuf-101, New salt pop. and Siwa populations (43.54%, 43.38%

and 43.35%, respectively). Root characters play an important role in alfalfa plant adaptation to biotic and abiotic stress. Root system size was the best indirect selection criterion for yield in alfalfa (Chloupek *et al* 1999 and Basafa and Taherian, 2009). Crown region was varied from centered (compact) and longest types of tested. Plant samples of Cuf-101, Salt America, Balady, Fixed-N, New valley-1 and New Valley-2 populations were compacted crown region with spaced ranges varied from 0.30 to 0.53 cm, while Siwa, Ismaelia-94, Ismaelia-1 and New Salt pop. populations were longest crown area with space regions varied from 2.03 to 1.67 cm. The average mean of crown area recorded 0.97 cm of selected plants and 0.91 cm of control populations. Ismaelia-1 showed the highest value (2.33cm) followed by Ismaelia-94 and Siwa populations (1.67 and 1.50 cm, respectively). Whereas, New valley-2 recorded the lowest value (0.30 cm) across all populations (Table 3).

The crown diameter varied from 2.33 to 3.60 cm with an average mean of 3.03 cm. New Salt pop., Ismaelia-1 and Siwa populations were the best crown root diameter (cm) across all populations (3.60, 3.60 and 3.50, respectively). This data was agreed with Oushy *et al* (2007) with minor differences. Branes and Sheaffer (1995) reported that alfalfa cultivars differ in crown type. The region space or length of the crown region may be correlated with crown tillers number and expected forage yield. Our study recorded high variation between alfalfa root system size among tested populations.

The New Salt population noted the highest number of lateral roots (20.33) across all populations but New valley-2 population recorded the lowest number of lateral roots (12.30). Lamb *et al* (2000b) mentioned to the importance of lateral roots and increase of herbage yield. Selecting plants for vigorous root morphology may have influenced herbage yield by inadvertently selecting for changes in disease resistance response or root size and weight.

It is of interest to overall selected populations mean after tested against the three fungal seedling damping-off diseases exceeded the mean of control over populations for number of tillers, leaf stem ratio (%), crown diameter and number of lateral roots. However, plant height (cm) and stem diameter (cm) were equally both of selected tolerant populations and their control parents (Table 3). Overall, this is suggested that healthy root system and lateral root vigor can improve plant tolerance against damping-off fungal stress.

Table 3. Means and total values agronomic characters for control and selected plants per populations of ten alfalfa more tolerant against the three pathogenic fungi over three cuts in field.

Populations	Shoot characters				Root characters		
	Plant height (cm)	Stem diameter (cm)	No. tillers	Shoot root %	Crown region (cm)	Crown diameter (cm)	No. lateral roots
Cuf-101	61.00 ab	0.53 a	20.67 a	43.54 a	0.53 c	3.20 c	16.00 bc
Salt America	59.00 ab	0.47 a	18.33 bc	38.96 b	0.50 c	3.07 c	18.03 ab
Balady	56.67 bcd	0.35 b	13.67 c	32.70 d	0.33 c	2.53 d	12.67 e
Fixed N	60.00 ab	0.50 a	16.00 bc	34.05 c	0.50 c	3.37 b	13.33 de
New valley 1	52.67 d	0.37 b	16.00 bc	32.73 d	0.50 c	2.37 d	12.67 e
New Valley 2	58.33 bc	0.36 b	15.67 bc	35.38 b	0.30 c	2.33 e	12.33e
Siwa	63.33 a	0.50 a	23.03 a	43.35 a	1.67 b	3.50 a	18.78 ab
Ismaelia 94	54.00 cd	0.47 a	17.00 b	32.81 cd	1.67 b	3.17 c	16.33 bc
Ismaelia 1	58.00 bc	0.50 a	20.67 a	38.47 ab	2.03 a	3.60 a	17.67 b
New Salt pop.	62.67 a	0.51 a	22.67 a	43.38 a	1.67 b	3.60 a	20.33 a
Selected pop. mean	**58.59**	**0.46**	**18.40**	**37.54**	**0.97**	**3.03**	**15.81**
Control mean	**57.76**	**0.47**	**16.73**	**36.83**	**0.91**	**2.57**	**12.67**

Means followed by the same letter are not significantly different at the P ≤ 0.05.

The result obtained in terms of dry yield was similar to that of fresh yield reduction with minor differences in the rank within alfalfa populations. The yield reduction (fresh and dry yield) of infected alfalfa plants may be related to either reduce percentage of stand plants or the deleterious effects of the fungal pathogens on plant growth (reducing shoot length, stem diameter or leaf area and root system vigor). Similar trend of the present study was previously reported by Zaidi (2003), who found a reduction in shoot and root length and dry mater (Tables 2 and 3).

So, selection for the healthy alfalfa plants across fungal pathogens infection in the field, between and within populations, produce promising genotypes more tolerant to seedling damping-off diseases.

Crude protein%

Crude protein % indicated significant differences (P≤0.05) across the infected populations and their controls. Table (4) shows that the highest crude protein (CP) % were existed in Siwa, New Salt pop., Ismaelia-94, Cuf-101 and Fixed N populations (21.25, 21.13, 20.63, 20.54 and 20. 42%, respectively). Crude protein content of infected alfalfa

populations were decreased from 0.28 % (Siwa) to 5.71% (New valley-1) with reduction mean of 2.19% across all populations. Results across populations recorded decreasing of crude protein content as a result of infection compared with control traits. Similar result was early reported by Llieva and Blazher (1995). Crude protein % of forage yield in alfalfa populations were generally negatively association (Elliot *et al* 1972). Accordantly, fungal reduced subsequently quality of alfalfa in terms of protein content.

Table 4. Crude protein (%) of ten alfalfa populations infected with pathogenic fungi compared with control treatment (un- infested soil).

Populations	Crude protein%		
	Un- infested soil	Infested soil	Reduction%
Cuf-101	20.97 ab	20.54 ab	**2.05**
Salt America	19.87 d	19.46 b	**2.06**
Balady	19.75 d	19.29 bc	**2.33**
Fixed N	20.65 bc	20.42 b	**1.11**
New valley 1	19.96 d	18.82 c	**5.71**
New Valley 2	19.68 d	18.86 c	**4.17**
Siwa	21.31a	21.25 a	**0.28**
Ismaelia 94	20.79 bc	20.63 a	**0.77**
Ismaelia 1	21.16 a	20.86 a	**1.42**
New Salt pop	21.41 a	20.93 a	**2.34**
Mean	**20.55**	**20.10**	**2.19**

Means followed by the same letter are not significantly different at the P ≤ 0.05.

Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

Protein markers, including electrophoresis protein and isozymes were among the first group of molecular markers exploited for genetic diversity assessment. The electrophoretic banding patterns of proteins extracted from leaves of 10 alfalfa populations non-stressed (control) are shown in Fig. (1) and their densitometric analysis are illustrated in Table (5). The similarity matrix of the seven commercial local (New valley-1, New valley-2, Balady, Siwa, Ismaelia -94, Ismaelia -1 and Fixed N,), two exotic population; (Cuf-101 and Salt America) and new promised population(New Salt pop.) represents in Table(6) and the relationships among them illustrates in Fig (2).

The electrophoretic banding patterns of proteins extracted from leaves of ten alfalfa populations stressed plants (grown in soil infested with three fungi causing seedling damping-off disease *(Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani*) in green house are shown in Figures (3), (4) and (5), respectively, and their densitometric analysis are illustrated in Tables (7), (8) and (9), respectively, where the presence and absence of bands were assessed with (1) and (0), respectively. The relationships among the ten alfalfa populations grown under fungal stress conditions are illustrate in Fig (6).

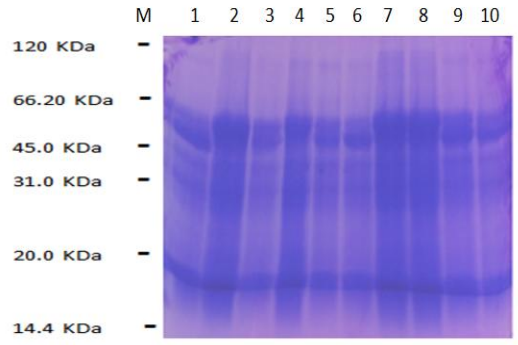


Fig. 1. SDS-protein banding patterns for ten alfalfa populations grown under non-stressed (normal condition). Where, 1 = New valley -1, 2 = New valley-2, 3 = Balady, 4 = Siwa, 5 = Salt America, 6= Cuf-101, 7= Ismaelia -94, 8= Ismaelia -1, 9= New Salt pop. and 10= Fixed N.

The results of SDS-PAGE revealed a total number of 15 bands with molecular weights (MW) ranging from about 15.5 to 115.1 KDa in ten alfalfa populations grown under normal conditions (non-stressed conditions), which were not necessarily present in all populations, as shown in Fig. (1) and conditions), which were not necessarily present in all populations, as shown in Fig. (1) and Table (5). There is no resemblance between any population and each other and a unique fingerprint characterized each. The variations among them might be due the differences in their genetic makeup. The SDS-protein banding patterns of the ten alfalfa populations was found to be useful in identifying the variation between them and identifying the finger print of each studied population.

Data showed two common bands (monomorphic) at MW of 40.3 and 16.9KDa, while the remaining bands were polymorphic with polymorphism percentage equal 86.7 under the control conditions.

Similarity indices and consensus tree were developed on the basis of the protein banding patterns of the ten alfalfa populations using protein banding patters (Fig. 1). The two most closely related populations were Ismaelia-94 and both of the commercial local population Ismaelia-1 and New Salt Pop. with the highest similarity index (0.947), as shown in Table (6).

Table 5. Densitometric analysis for SDS leaves storage protein (water soluble fraction) of ten alfalfa populations under stress non-stressconditions (control)

No of band	MW (KDa)	1	2	3	4	5	6	7	8	9	10
1	115.1	0	0	0	1	0	1	1	1	1	0
2	93.5	1	1	0	1	1	0	1	1	1	0
3	64.6	0	0	0	0	1	0	0	0	0	1
4	59.4	1	1	1	1	0	0	1	1	1	0
5	56.1	0	0	1	0	1	1	0	0	0	1
6	53.0	0	0	1	1	0	0	1	1	1	0
7	50.6	1	1	0	0	0	0	0	0	1	1
8	49.2	0	0	0	0	1	1	1	1	1	0
9	40.3	1	1	1	1	1	1	1	1	1	1
10	34.0	1	1	1	1	0	0	1	1	1	1
11	30.9	0	1	0	1	1	1	0	0	0	1
12	29.2	1	1	0	1	0	1	1	0	0	1
13	18.9	0	0	1	0	0	1	1	1	1	0
14	16.9	1	1	1	1	1	1	1	1	1	1
15	15.5	1	0	1	0	0	0	0	0	0	0
Total number of bands		8	8	8	9	7	8	10	9	10	8

Where,
1 = New valley -1, 2 = New valley-2, 3 = Balady, 4 = Siwa, 5 = Salt America, 6= Cuf-101, 7= Ismaelia -94, 8= Ismaelia -1, 9= New Salt pop. and 10= Fixed N

This highest similarity index followed by the similarity index between Ismaelia-94 and New Salt Pop., which was recorded 0.900. On the other hand, the two most distantly related populations were Ismaelia-94 and Fixed N with low similarity index (0.353), followed by the similarity index between New valley-1 and Cuf-101 (0.375). The similarity index recorded 0.400 between Salt America and both of New Valley-1 and Balady, as shown in Table (6).

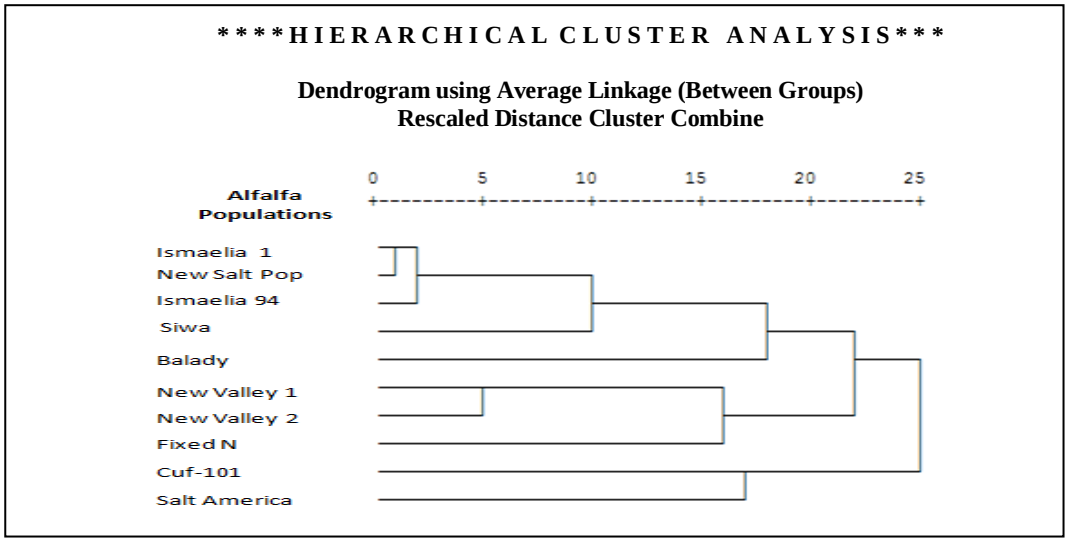
The results of the consensus tree (Fig. 2) indicated that tree was divided the ten alfalfa populations into two main clusters, the first included the two exotic populations Salt America and Cuf-101. The second main cluster was divided into two sub-clusters. The first sub-cluster was divided into two sub-sub-clusters. The first one included Fixed N alone, while the other included the two commercial local populations: New Valley-1 and New Valley-2. The second sub-cluster was divided into two sub-clusters. The first one was included commercial local Balady populations alone, while the second one was divided to separate Siwa alone in sub-sub cluster, while the other one was included the rest of the commercial local population that grouped finally Ismaelia- 94, New Salt Pop. and Ismaelia- 1 in the same group, as they recorded the highest similarity index, as shown in Table (6) and Fig. (2).

The results of SDS-PAGE of the ten alfalfa populations grown under disease stress conditions (*Fusarium oxysporum*) revealed a total number of 14 bands (with molecular weights (MW) ranging from about 12.2 to 81.0 KDa, as shown in Fig (3) and Table (7).

Table 6. Similarity matrix among the ten alfalfa populations based on Protein banding patterns analysis.

Alfalfa populations	1	2	3	4	5	6	7	8	9
2	0.875								
3	0.625	0.500							
4	0.706	0.824	0.588						
5	0.400	0.533	0.400	0.500					
6	0.375	0.500	0.500	0.588	0.667				
7	0.667	0.667	0.667	0.842	0.471	0.667			
8	0.588	0.588	0.706	0.778	0.500	0.588	0.947		
9	0.667	0.667	0.667	0.737	0.471	0.556	0.900	0.947	
10	0.625	0.750	0.500	0.588	0.667	0.625	0.444	0.353	0.444

Where,
1 = New valley -1, 2 = New valley-2, 3 = Balady, 4 = Siwa, 5 = Salt America, 6= Cuf-101, 7= Ismaelia -94, 8= Ismaelia -1, 9= New Salt pop. and 10= Fixed N.



Where, 1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8= Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

Data showed two common bands (monomorphic) at the Molecular weight of 45.4 and 36.7KDa, while the remaining bands were polymorphic with 88% polymorphism under the stress conditions. Regarding with the data of the reaction of the ten alfalfa populations against seedling damping-off disease caused by *Fusarium oxysporum*, under greenhouse conditions in Table (1), the band number 5 with molecular weight of 31.6 KDa was detected as positive marker for resistance against damping-off caused by *Fusarium oxysporum*, while this band was observed in each of Cuf-101, Salt America and New Salt Pop that were recorded as resistant populations to damping-off caused by *Fusarium oxysporum* (Table 1), while band number 3 with molecular weight of 42.6 KDa was detected as positive marker for susceptibility against damping-off caused by *Fusarium oxysporum*, as well as, band number 11 with molecular weight of 18.3 KDa was detected as positive marker for moderate susceptibility against damping-off caused by *Fusarium oxysporum*, and band number 6 with molecular weight of 29.4 KDa was detected as positive marker for highly susceptibility against damping-off caused by *Fusarium oxysporum*, as shown in Table (7).

The occurrence of new bands and absence of others under fungal stress conditions with *Fusarium oxysporum* compared with the protein banding patterns under non-stressed condition (control) represented by different alfalfa populations would indicate either enhancement or repression of gene expression in these populations.

Table 7. Densitometric analysis for SDS leaves storage protein (water soluble fraction) of ten alfalfa populations grown on *Fusarium oxysporum* disease stressed condition.

No of band	MW (KDa)	1	2	3	4	5	6	7	8	9	10
1	**81.0**	0	1	1	0	1	1	1	1	0	0
2	**45.4**	1	1	1	1	1	1	1	1	1	1
3	**42.6**	0	0	0	0	0	0	0	1	0	0
4	**36.7**	1	1	1	1	1	1	1	1	1	1
5	**31.6**	1	1	0	0	0	0	0	0	0	1
6	**29.4**	0	0	1	1	1	1	0	0	0	0
7	**25.6**	0	0	0	0	1	0	0	0	0	0
8	**24.8**	0	0	0	0	0	0	0	1	1	1
9	**23.3**	0	1	1	1	1	1	1	0	0	0
10	**20.2**	1	1	1	0	1	1	1	1	1	1
11	**18.3**	0	0	0	0	0	0	1	0	1	0
12	**16.6**	0	0	0	0	0	0	0	1	1	1
13	**13.4**	0	0	0	0	0	1	1	1	0	0
14	**12.2**	0	0	0	0	0	0	0	1	1	0
Total number of bands		**4**	**6**	**6**	**4**	**7**	**7**	**7**	**9**	**7**	**6**

Where,
1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8= Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

This may alter the produced proteins in response to pathogen stress either on the transcriptional or post transcriptional levels of gene expression. Also, there is a flow of information from the genes of an organism into the construction of specific proteins, generally referred to as gene expression; it is the spectrum of proteins produced that provides the connection between genotype and phenotype. Many environmental factors are now known to greatly influence the extent to which specific genes are activated to produce proteins that are protective and which enable organisms to survive (Burdon, 1999).

The results of SDS-PAGE of the ten alfalfa populations grown under disease stress conditions infected with *Macrophomina phaseolina*, revealed a total number of 16 bands, which were not necessarily present in all populations, with molecular weights (MW) ranging from about 8.8 to 89.6 KDa as shown in Fig (4) and Table (8).

Data showed three common bands (monomorphic), while the remaining bands were polymorphic with 81% polymorphism under the stress conditions. Regarding with the data of the reaction of the ten alfalfa populations against seedling damping-off disease caused by *Macrophomina phaseolina*, under greenhouse conditions in Table (1), the bands number 1, 10, 12 and 13 with molecular weight of 89.6, 20.2, 16.3 and 13.0 KDa, respectively were detected as positive markers for resistance against seedling damping-off caused by *Macrophomina phaseolina*, where these bands were observed in each of Salt America and New Salt Pop that were recorded as resistant populations to seedling damping-off caused by *Macrophomina phaseolina* (Table 1), while band number 6 with molecular weight of 29.7 KDa was detected as negative marker for moderate susceptibility against damping-off caused by *Macrophomina phaseolina*, as shown in Table (8).

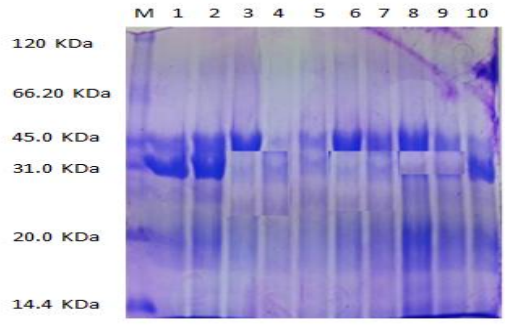


Fig. 3. SDS-protein banding patterns for ten alfalfa populations grown under stressed condition (grown on *Fusarium oxysporum*).
Where,
1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8=Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

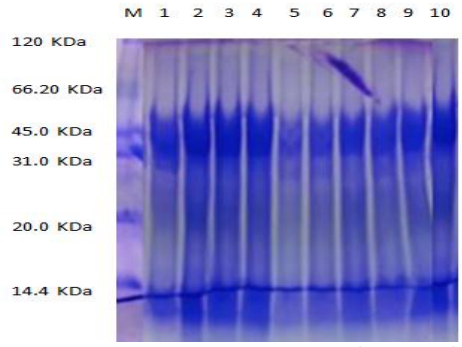


Fig. 4. SDS-protein banding patterns for ten alfalfa populations grown under stressed condition (grown on *Macrophomina phaseolina*).
Where, 1 = Cuf-101, 2 = Salt America, 3 = Balady, 4= Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8= Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

For the ten alfalfa populations grown under disease stress conditions infected with *Rhizoctonia solani*, the data of SDS-PAGE revealed a total number of 12 bands, which were not necessarily present in all populations, with molecular weights (MW) ranging from about 12.9 to 59.3 KDa as shown in Fig (5) and Table (9).

Data showed three common bands (monomorphic) at the molecular weight of 59.3, 48.5 and 12.9 KDa, while the remaining bands were polymorphic with 75% polymorphism under the *Rhizoctonia solani* stress conditions. Regarding with the data of the reaction of the ten alfalfa populations against seedling damping-off disease caused by *Rhizoctonia solani*, under greenhouse conditions in Table (1), band number 7 with molecular weight of 30.9 KDa was detected as positive marker for susceptibility against damping-off caused by *Rhizoctonia solani*, while band number 9 with molecular weight of 26.0 KDa was detected as negative marker for susceptibility against damping-off caused by *Rhizoctonia solani*, these bands were observed in each of Cuf-101, Salt America and New Salt Pop that were recorded as susceptible populations to damping-off caused by *Rhizoctonia solani* (Table 1), also band number 6 with molecular weight of 33.3 KDa was detected as positive marker for highly susceptibility against damping-off caused by *Rhizoctonia solani*, as shown in Table (9).

Consensus tree was developed on the basis of the combined protein banding patterns of the ten alfalfa populations grown under all fungal stress conditions under this investigation *(Fusarium oxysporum, Macrophomina phaseolina and Rhizoctonia solani*), (Fig. 6). The results of the consensus tree indicated that tree was divided the ten alfalfa populations into two main clusters, the first included the two most resistant populations: Salt America and the new promised population (New Salt Pop.). The second main cluster was divided into two sub-clusters. The first sub-cluster included the exotic.

Table 8. Densitometric analysis for SDS leaves storage protein (water soluble fraction) of ten alfalfa populations grown on *Macrophomina phaseolina* disease stressed condition.

No of band	MW (KDa)	1	2	3	4	5	6	7	8	9	10
1	89.6	0	1	0	0	0	0	0	0	0	1
2	47.9	1	1	1	1	1	1	1	1	1	1
3	43.9	0	1	0	0	0	0	0	1	1	1
4	39.2	1	1	1	1	1	1	1	1	1	1
5	35.4	1	1	0	0	0	0	0	1	0	1
6	29.7	0	1	1	1	1	1	0	1	0	1
7	27.2	1	1	1	1	0	0	0	0	0	1
8	24.3	0	1	1	1	0	1	1	0	0	1
9	22.3	0	1	1	1	0	0	0	0	1	1
10	20.2	0	1	0	0	0	0	0	0	0	1
11	17.6	0	0	1	1	0	0	0	0	0	0
12	16.3	0	1	0	0	0	0	0	0	0	1
13	13.0	0	1	0	0	0	0	0	0	0	1
14	10.8	1	1	1	1	1	1	1	1	1	1
15	9.0	1	1	1	1	0	0	0	0	1	1
16	8.8	0	0	0	1	0	0	0	0	0	0
Total number of bands		6	14	9	10	4	5	4	6	7	14

Where,

1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8= Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

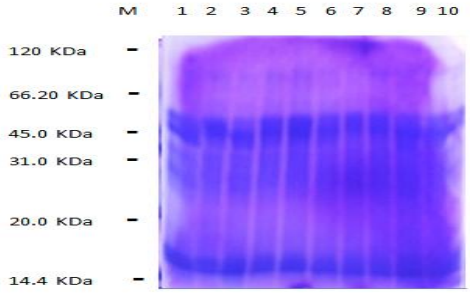


Fig. 5. SDS-protein banding patterns for ten alfalfa populations grown under stressed condition (grown on *Rhizoctonia solani*).

Where,

1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8= Ismaelia -94, 9= Ismaelia -1 and 10= New Salt pop.

The development of disease resistance was found to be correlated with the accumulation of host synthesized new polypeptides (Broglie *et al*., 1986). Hlinkova and Sykora (1996) recorded that the new protein contents depended on host genotype and virulence genes of the pathogens. Proteins with peroxidase activity differed at the level of susceptibility host-pathogen interaction. Radwan (2000) detected new proteins in the mutagenized resistant and immune plants of barely to powdery mildew (new genotypes) from the two tested cultivars in comparison with the susceptible mutant ones and the control (parents). The changes of proteins depended on the host genotype and sensitivity to infection.

Table 9. Densitometric analysis for SDS leaves storage protein (water soluble fraction) of ten alfalfa populations grown on *Rhizoctonia solani* disease stressed condition

No of band	MW (KDa)	1	2	3	4	5	6	7	8	9	10
1	59.3	1	1	1	1	1	1	1	1	1	1
2	48.5	1	1	1	1	1	1	1	1	1	1
3	43.3	0	0	1	1	1	1	0	0	1	0
4	41.6	0	0	0	0	0	0	1	1	0	1
5	38.1	0	1	1	1	1	0	1	0	1	1
6	33.3	0	0	1	1	1	1	0	1	1	0
7	30.9	1	1	0	0	0	0	1	0	0	1
8	28.7	0	1	1	0	0	1	0	1	1	1
9	26.0	0	0	1	1	1	1	0	1	1	0
10	15.9	0	1	0	0	0	0	1	0	0	0
11	13.3	0	0	1	1	1	1	1	1	1	1
12	12.9	1	1	1	1	1	1	1	1	1	1
Total number of bands		4	7	9	8	8	8	9	8	9	7

Conclusions

The choice of resistant populations seems to be very important to improve the forage yield, the agronomic technique, harvest frequency, selection within and between population and breeding for seedling damping- off root diseases resistant are the major factors to improve alfalfa forage and quality yield.

A clear difference between the local populations was obtained with respect to forage and dry matter yield. Balady achieved a lower level, while New valley-1 and New valley-2 showed an intermediate level of forage and quality yield potential (crude protein %). The New Salt pop. characterized by a good forage and protein content, with same performance of Siwa population followed by Ismaelia-1, Ismaelia-94 and Fixed N populations, respectively.

Variation in alfalfa plants and populations of their susceptibility and/or resistance to seedlings damping-off disease depend mainly on the virulence pathogenic fungi and plant genotype. Salt America and New Salt pop., populations were more resistant to seedlings damping-off fungal disease.

Individual selection, between and within populations, for shoot forage yield, root vigors and healthy plants produce a new promising population more resistant to seedlings damping-off disease.

The results of SDS-PAGE revealed that there is no resemblance between any studied population and each other and a unique fingerprint characterized each, which facilitate the development of each population`s fingerprint. The two most closely related populations were Ismaelia 94 and both of the two commercial local populations Ismaelia 1 and Siwa with the highest similarity index (0.947), on the other hand, the two most distantly related populations were the new promised population (New Salt Pop.) and Ismaelia 94 with low similarity index (0.353). Data showed that the band with molecular weight of 31.6 KDa was detected as positive marker for resistance against damping-off caused by *Fusarium oxysporum*, while this band was observed in each of Cuf-101, Salt America and New Salt Pop that were recorded as resistant populations to damping-off caused by *Fusarium oxysporum.*

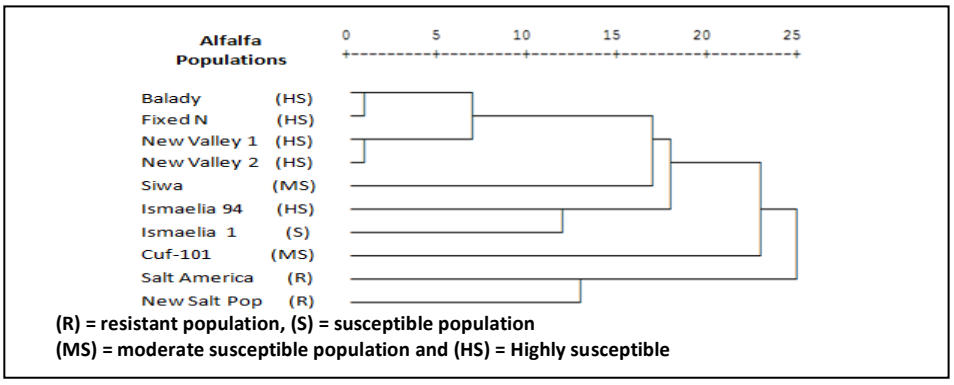


Fig. 6. Dendrogram showing the genetic distance among the ten alfalfa populations under fungal stress conditions using SDS- protein data.

Where, 1 = Cuf-101, 2 = Salt America, 3 = Balady, 4 = Fixed N, 5 = New valley -1, 6= New valley-2, 7= Siwa, 8=Ismaelia-94, 9= Ismaelia-1 and 10= New Salt pop.

The occurrence of new bands and absence of others under fungal stress conditions with *Fusarium oxysporum* compared with the protein banding patterns under non-stressed condition (control) represented by different alfalfa populations would indicate either enhancement or repression of gene expression in these populations.

The bands with molecular weight of 89.6, 20.2, 16.3 and 13.0 KDa were detected as positive markers for resistance against damping-off caused by *Macrophomina*, while these bands were observed in each of Salt America and New Salt Pop that were recorded as resistant populations to damping-off caused by *Macrophomina phaseolina.*

Consensus tree was developed on the basis of the bulked protein banding patterns of the ten alfalfa populations grown on three fungi causing seedling damping-off disease (*Fusarium oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani*) in greenhouse. The dendrogram gathered the resistant populations in one cluster and almost all highly susceptible populations together in the same group.

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