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Alleviation of salinity stress in faba bean (*Vicia faba* L.) plants by inoculation with plant growth promoting rhizobacteria (PGPR)

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Abstract

The effects of three bacterial isolates (*Pseudomonas putida*, *P. fluorescens* and *Bacillus subtilis*) of plant growth promoting rhizobacteria were investigated to improve the growth of six cultivars from *Vicia faba* L. under two levels of salinity stress (S_1=4000 and S_2=8000 ppm). Different morphological and biochemical parameters were studies. The highest values of final germination percent were observed in cultivar Wadi 1 and Line 1 (96%) in the presence of *P. fluorescens*. Application of *P. putida* decreased the mean germination time in the presence or absent salinity stress. The salinity treatments induced a significant decreased in all of plant growth parameters, plant chlorophyll and plant soluable proteins, while plant proline was increased, comparing to control. The plants treated with *P. fluorescens* showed significant increase in growth traits such as plant length (10.66%), plant shoot fresh weight (9.52%) and plant leaf area (61.86%). This increasing trend was followed by application of *B. subtilis* then *P. putida.* The phylogenetic diversity relationship and the level of genetic diversity among the cultivars were assessed using 9 arbitrary primers. The results indicated that 6 out of the 9 primers [(GATA)4GC, (AGAC)4GC, GAC(GATA)4, (AC)8YC, CGC(GATA)4 and (AG)8YC] can generate clear multiplex banding profiles. Among the 48 polymorphism bands, only 14 were found to be useful as positive or negative markers related to salt stress. The cluster analysis classified the cultivars into two main groups. The inter-simple sequence repeat can be used to identify alleles associated with the salt stress in faba bean germplasm.

Keywords: Biochemical markers; Biodegradation; ISSR markers; Rhizobacteria; Salt stress.
Abbreviations: CFU_Cell forming unit; cm_centimeter; cv._cultivar; FGP_final germination percent; FW_fresh weight; g_gram; ISSR_inter simple sequence repeat; mg_milligram; MGT_mean germination time; PGPR_plant growth promoting rhizobacteria; ppm_part per million; S_salinity level; UPGHA_un-weighted pair group method with arithmetic hean.

Introduction

Salinity is the main threat to the plant production in many countries all over the world (Munns and Tester, 2008). The main factor for increasing soil salinity is irrigation of plant with saline water, poor cultural practices, and low precipitation. Irrigated agriculture consumes about 90 % of the total water withdrawal to produce 36 % of the global food (Rengasamy, 2006). Also, salinity occurred from irrigation is widely responsible for increasing the concentration of dissolved salts in the soil profile to a level that impairs plant growth and results in abandoning agricultural lands (Munns, 2005; Egamberdiyeva et al., 2007; Manchanda and Garg, 2008). The interest in sustainable agriculture has drawn attention to some microbes that can be beneficial. These microbes might be neutral or even pathogenic. Beneficial rhizobacteria can improve seed germination, root and shoot growth, nutrient uptake, and plant stress tolerance (Lugtenberg and Kamilova, 2009). Moreover, they are able to control various diseases. They are often referred to as plant growth-promoting rhizobacteria (PGPR) (Hiltner, 1904; Lugtenberg et al., 2001; Compant et al., 2005; Arora et al., 2008; Lugtenberg and Kamilova, 2009). So far, a range of salt-tolerant rhizobacteria (e.g., *Rhizobium*, *Azospirillum*, *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, and *Bacillus*) has shown beneficial interactions with plants in stressed environments (Adesemoye et al., 2008; Egamberdieva and Islam, 2008; Egamberdieva et al., 2011; Almagrabi et al., 2014). The yield of crops such as bean, corn and onion can be reduced by 50 %, when the soil EC is increased to 5.0 dS/m (Horneck et al., 2007). Plants may protect themselves from drought and salt stress by accumulating compatible solutes such as sugars and amino acids to osmotically adjust. Bano et al. (2013) observed that *A. lipoferum* increased accumulation of free amino acids and soluble sugars in plants under drought stress conditions. *Azospirillum* inoculation leads to an increased content of proline (Kandowangko et al., 2009) and free amino acids in maize plants under drought stress conditions (Barka et al., 2006; Sziderics et al., 2007; Sandhya et al., 2010), enhance proline synthesis in stressed plants, which helps in maintaining the cell water status; thereby, helping the plant to cope with the salinity stress. To expand the plant growing in a wide range of environments and to increase the yield stability in terms of quantity and quality

under a certain growing conditions, resistance to abiotic stress is important. This goal can be achieved through conventional plant breeding complemented with various biotechnology developments to distinguish between different plant cultivars and select the best tolerant cultivars (Damude and Kinney, 2008). With the development of molecular biology tools, genetic variation can now be identified at the molecular level based on changes in the DNA and their effect on the phenotype instead of visual selection. Nowadays, the plant breeding has become quicker, easier, more effective, stable and more efficient (Phillips, 2006). One of the most efficient molecular tools is inter simple sequence repeat (ISSR). It is a PCR technique that uses repeat-anchored or non-anchored primers to amplify DNA sequence between two inverted SSRs (Zietkiewicz et al., 1994; Nybom 2004). ISSR markers have been successfully used for the assessment of genetic diversity in corn or bean (Galvan et al., 2001; Akram and Kianoosh, 2012; Shlvakum and Subramanya, 2014). ISSR is quick, easy to handle, highly reproducible, can yield reliable and reproducible bands, and the cost of the analysis is relatively lower than that of some other markers. It is highly polymorphic fingerprints with enough resolution to distinguish genotypes within a relative narrow range of genetic diversity (Sandra et al., 2014; Bornet and Branchard, 2001; Guo et al., 2009). ISSRs are better tools than RAPDs to identify beans by gene pool of origin though they did not reveal as many differences between individuals as RAPDs (Galvan et al., 2003). In this study, we surveyed the effects of some isolates of PGPR on morphological and physiological traits for six cultivars from faba bean plants growing under salinity stress at *in vitro* and/or green house condition. In addition, molecular markers (ISSR-PCR) associated with salt tolerance in plant and the levels of genetic diversity relationship among genotypes were assessed. This relationship could be used by breeder to establish strategies for selecting early generation materials in faba bean breeding programs.

Results

Effect of PGPR on faba bean germination rate and seedling growth in-vitro

The effect of two levels of salinity and three isolates of PGPR on different faba bean cultivars on final germination percent (FGP) and mean germination time (MGT) was shown in Table 3. The highest value of FGP (92 %) was recorded in faba bean cv. Wadi1 followed by Line 1 (90%), Line 1706 (85%), Line 137 (84%) then cv. Misr 3 (83%) and cv. Giza 2 (72%). The treatment of salinity in two levels (S_1= 4000 ppm, S_2= 8000 ppm) caused decrease in values of FGP in all different genotypes. The lowest value of FGP (54%) was found when plant treated with salinity level S_2. In the presence of salt FGP decreased by 25% in plant cv. Giza 2, while the three plant cultivars Wadi 1, Line 137 and Line 1 exhibited the best result in the second level of salinity treatment (78%, 70% and 66%, respectively). The treated plants with the three isolates of PGPR were recorded increase in FGP in all faba bean varieties, comparing with control treatment. The highest values of FGP in the presence of PGPR treatment were observed in faba bean cultivars Wadi 1 and Line 1 (96%) when treated with *Pseudomonas fluorescens* ($PGPR_2$). The beast results were obtained from the combination treatment between salinity stress level S_1 and PGPR in the following four cultivars Wadi 1 (90%), Line 1 (88%), Line 137 (86%) and Giza 2 (78%), when the plant treated with *P. fluorescens* ($PGPR_2$). The *Bacillus subtilis* ($PGPR_3$) has given a good result under salinity stress level S1 for two cv. Misr3 (86%) and Line 1706 (84%), comparing with other treatments. Also *B. subtilis* ($PGPR_3$) was distinguished as the best bacterial strains for decreasing the deficiency effect for salinity stress in level S_2 for FGP values, comparing with other treatments and control. The same effects of two salinity stress levels were found in MGT values that increased the MGT, comparing with control. The bacterial isolate *P. putida* ($PGPR_1$) was more effective for decreasing the values of MGT in the presence or absent of salinity stress levels (S_1 and S_2), that suggests it as the best bacterial isolates for plant growth promoting used in this study.

Effect of PGPR on faba bean growth and biochemical traits in greenhouse

The effect of three isolates of PGPR on some of plant growth traits for six cultivars of faba been in the presence of salinity is shown in Table 4. In general, the lowest values of all plant traits such as plant shoot length (cm), plant shoot fresh weight (g) and plant leave area (cm) were recorded in the plants treated with two concentration of water salinity, comparing with control or other treatments without salinity stress. The second level of salinity treatment (S_1= 4000 ppm) was more effective to reduce plant growth values than the first concentration of salinity treatment (S_2= 8000 ppm). The plant cultivars Misr 3 and Giza 2 were the most cultivars affected by the two salinity levels. The decreased rate in plant shoot length ranged between 56.69% for S1, 57.47% for S2 and 55.85 for S_1 and 57.5% for S_2 to both cultivars Misr 3 and Giza 2, respectively. The cultivar Wadi 1 followed by Line 1706, line 1 then Line 137 were observed as more tolerant under both salinity concentrations. The similar results were recorded in plant shoot fresh weight and plant leave area in the presence of salinity concentration solo. In contrast, all plant traits were increased when plant treated with the three PGPR comparing with plant free from bacteria. The maximum values in the three plant growth parameters were observed in faba been cultivar Wadi 1 after *Pseudomonas fluorescens* ($PGPR_2$) treatment. The treated plant with *P. fluorescens* increased plant traits by 10.66% for plant length, 9.52% to plant shoot fresh weight and 61.86% for plant leave area comparing with control values in each plant parameter. *Bacillus subtilis* ($PGPR_3$) was ranked in the second place for effects on plant growth parameters (10%, 0.73% and 42.8%) followed by *P. putida* ($PGPR_1$) (2%, 0.14% and 32.08%). In addition to the sole treatments, combination of three PGPR isolates reduced effect of salinity stress on all plant growth traits. The plant treated with isolate of *P. putida* ($PGPR_1$) with salinity level S_1 (4000 ppm) recorded the maximum plant growth parameters in all faba bean cultivars. However, in the second level of salinity S_2 (8000 ppm) the higher values of plant growth traits were found in the plant treated with *B. subtilis* ($PGPR_3$). The best result in plant growth traits was observed in cultivar Wadi 1 followed by Line 1706 then Line 1, in the presence of PGPR and salinity stress.

Fig. 1 shows the soluble protein content (mg g^{-1} FW) in plant leaves of faba bean cultivars in the presence of three PGPR isolates and two level of salinity. The two salinity levels (S_1 and S_2) decreased the plant soluble protein content. The maximum decrease in soluble protein was found in cv. Giza 2, while the minimum decrease were found in Wadi 1, Line 1 followed by Line 137, Line 1706 then Misr 3.

Table 1. Faba bean genotypes, source and pedigree.

No.	Genotype	Source	Pedigree
1	Line 137	*FCRI	Selected from Misr 1 (123A/45/76XG.3)×(62/1570/66×G.2)×(Romi×Habashi)
2	Line 1	FCRI	Selected from Misr 1(123A/45/76XG.3)×(62/1570/66×G.2)×(Romi×Habashi)
3	Line 1706	FCRI	Cross 998* Giza 461
4	Wadi 1	FCRI	Giza blanka * Triple white
5	Giza 2	Egypt	Selected from landraces
6	Misr 3	FCRI	Misr1[(123A/45/76XG.3)×(62/1570/66×G.2)×(Romi×Habashi)]*(kahera241*Giza461)

* FCRI Field Crop Research Institute, Agriculture Research Center, Cairo, Egypt.

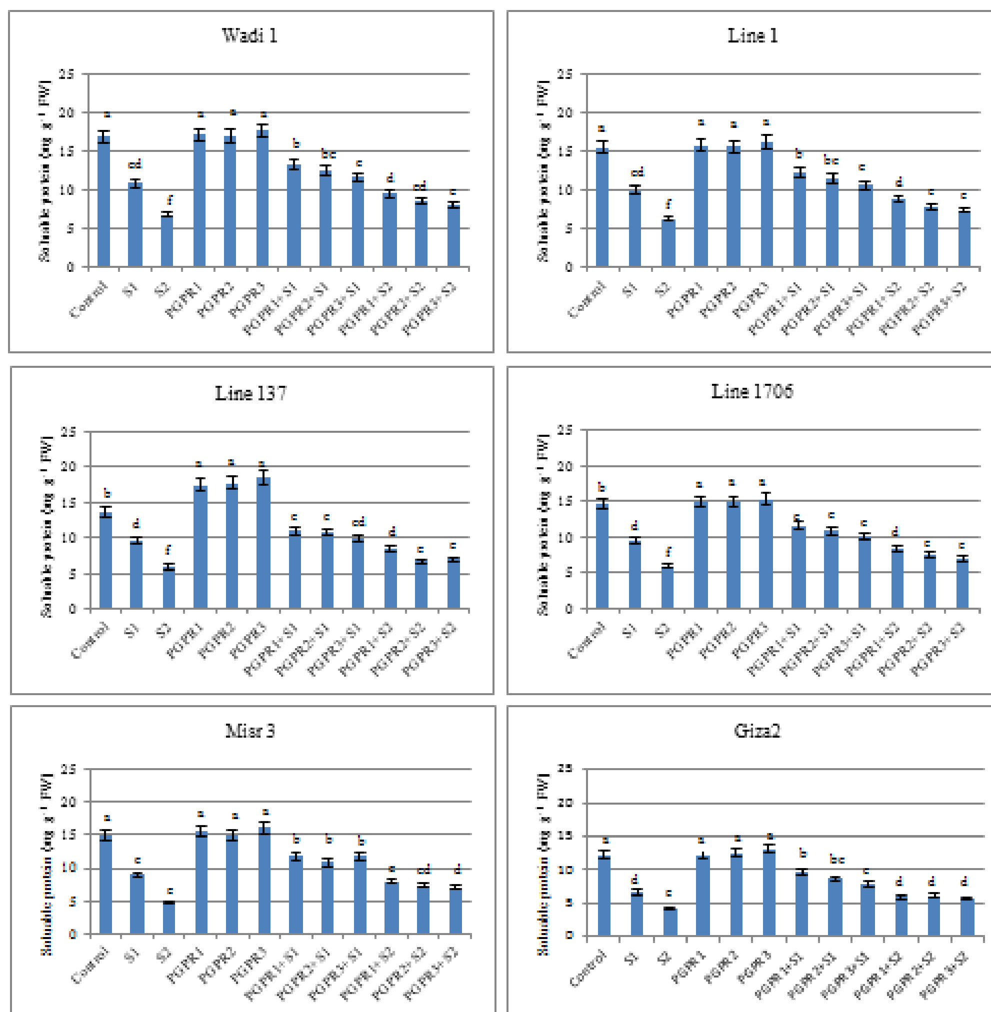

Fig 1. The soluable protein content (mg g^{-1} FW) in plant leaves for six cultivars of faba bean treated with three PGPR isolates in the presence of two level of salinity. *Pseudomonas putida* ($PGPR_1$), *P. fluorescens* ($PGPR_2$) and *Bacillus subtilis* ($PGPR_3$) - Salinity level: S_1= 4000 and S_2= 8000.

The plants treated with any isolates of PGPR recorded the increase in soluble protein comparing with non-inoculated plants (control). *Bacillus subtilis* ($PGPR_3$) was the best isolate between the three PGPR for increasing the plant soluble protein. The inoculation plants with *P. putida* ($PGPR_1$) recorded the best result in the presence of two level of salinity, comparing with other PGPR isolates or salinity treatments.

The data illustrated in Fig. 2 presented the proline content (ug g^{-1} FW) in plant leaves of faba bean varieties, treated by three PGPR in the presence of two salinity levels. The salinity stress increased the plant proline content, comparing with control or other treatment without salinity. The highest values for proline content were found in faba bean cultivars Wadi 1 (27.2 ug g^{-1} FW/S_2), Line 1706 (24 ug g^{-1} FW/S_1), Line1 (18.4 ug g^{-1} FW/S_1) followed by Line 137(16.23 ug g^{-1} FW/S_2) then Giza 2 (14.6 ug g^{-1} FW/S_2) and Misr 3 (13.80 ug g_{-1} FW/S_2). On the other hand, the three PGPR isolates were increased the plant proline but less than the effect of salinity levels, comparing with control. The interaction between salinity stress and PGPR caused a slight change in plant proline value in different faba bean varieties.

Fig. 3 discusses the effect of the three PGPR and two levels of salinity on plant chlorophyll content (µg cm^{-2}) in different faba been cultivars. Both salinity stress levels showed a negative effect on plant chlorophyll content, which were decreased sharply in salinity level S_2, when they compared to S_1 level and the plant control. The treatment of *B. subtilis*

Table 2. Code and sequence of ISSR primers.

Primer codes	Sequences	Primer codes	Sequences	Primer codes	Sequences
INC1	$(AG)_8YC$	INC4	$(Ac)_8YG$	INC7	$GAC(GATA)_4$
INC2	$(AG)_8YG$	INC5	$(GT)_8YG$	INC8	$(AGAC)_4GC$
INC3	$(AC)_8YT$	INC6	$CGC(GATA)_4$	INC9	$(GATA)_4GC$

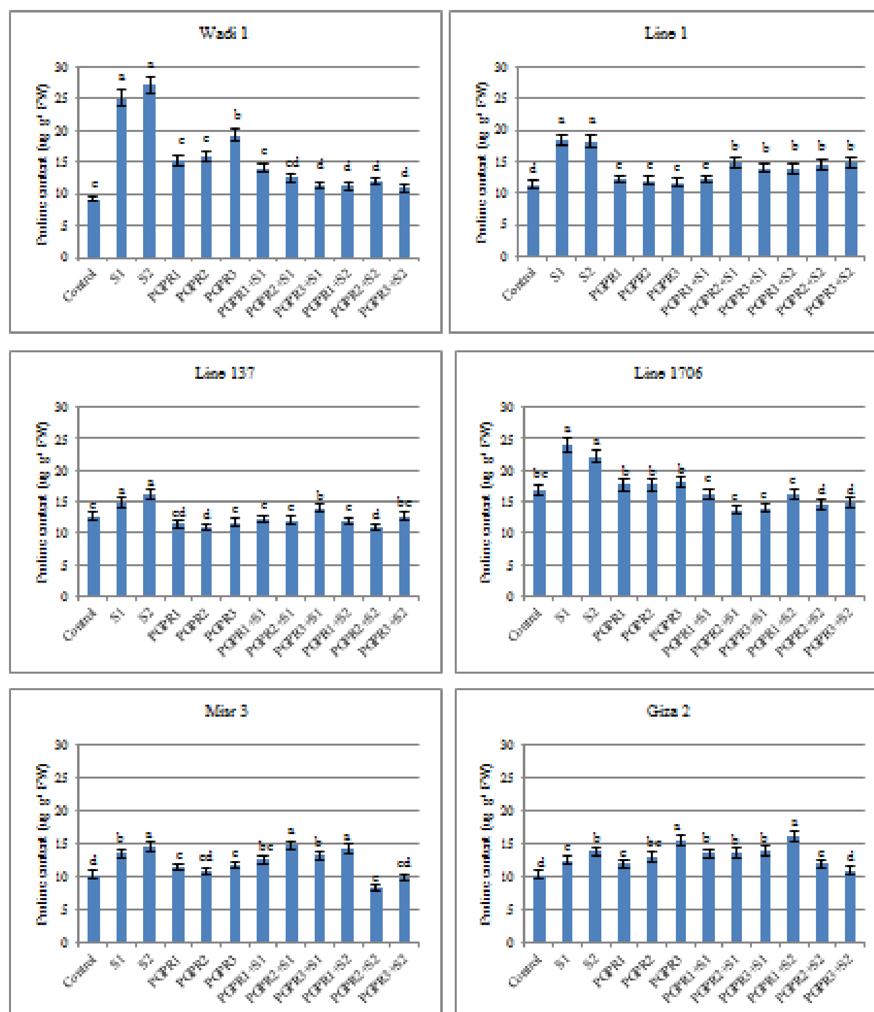

Fig 2. The proline content (ug g^{-1} FW) in plant leaves of six faba bean cultivars with treated by three PGPR in the presence of two level of salinity - Pseudomonas putida ($PGPR_1$), P. fluorescens ($PGPR_2$) and Bacillus subtilis ($PGPR_3$) - Salinity level: S_1= 4000 and S_2= 8000 ppm

($PGPR_3$) increased the plant chlorophyll content by 8.08% in Giza 2, 7.15% in Misr 3, 6.38% in Line 1, 5.48% in Wadi 1, 4.43% in Line 1706 and 0.94% in line 137, comparing with control values. All three PGPR isolates have given support for faba bean varieties under salinity stress condition by saving them from leaves discolors especially in the S_2 level.

Molecular study on faba bean plant varieties under salinity stress

The presented study illustrates how to assess phylogenetic diversity relationship and determine the level of genetic diversity among the faba bean cultivars using 9 arbitrary primers. All primers revealed various banding patterns with different polymorphism percentage (Table 5). A total of 91 alleles were separated by electrophoresis on agarose gel, ranging from 150 to 2500 bp in size. Out of the 91 scorable fragments, 48 were polymorphic, revealing 52.75% and the rest were monomorphic (47.25%), across the 6 studied cultivars. The highest number of bands (17) and polymorphism (70.5%) were generated with primer INC9 with sequence (GATA)4GC compared to other ISSR primers, while the lowest number of bands (4) and polymorphism (8.4%) were observed for primer INC2 and INC4 with sequence (AG)8YG and (AC)8YG, respectively.

The highest number of amplicons was generated in cv. Line 1 (68 amplicons), while cv. Line137 generated the lowest (55 amplicons) with different polymorphism locus percentage. Our study showed low polymorphism level values 47.25% among genotypes examined. A great deal of polymorphism was arbitrary; however, 14 bands were found to be useful markers related to salty stress (9 positive and 5 negative) (Table 6). When oligonucleotide INC1 was used, it produced

Table 3. The effect of two levels of salinity and three isolates of PGPR on six faba bean cultivars on final germination percent (FGP) and mean germination time (MGT).

Parameters	Treatments	Faba bean varieties					
		Line 137	Line 1	Line 1706	Wadi 1	Giza 2	Misr 3
Final germination percent (FGP)	Control	84 ± 3.78	90 ± 5.97	85 ± 3.61	92 ± 3.62	72 ± 2.74	83 ± 3.54
	S_1	76 ± 1.00	74 ± 4.76	70 ± 2.48	76 ± 3.11	62 ± 3.73	72 ± 2.74
	S_2	70 ± 2.08	66 ± 4.16	60 ± 1.51	78 ± 1.32	54 ± 2.33	62 ± 3.11
	$PGPR_1$	90 ± 1.52	92 ± 6.12	86 ± 2.96	94 ± 2.82	78 ± 1.65	88 ± 2.79
	$PGPR_2$	92 ± 2.64	96 ± 5.12	90 ± 2.15	96 ± 5.11	80 ± 3.41	92 ± 2.62
	$PGPR_3$	94 ± 1.00	92 ± 6.05	88 ± 3.48	92 ± 3.02	82 ± 3.63	90 ± 4.23
	$PGPR_1+S_1$	82 ± 2.64	86 ± 4.61	80 ±1.00	84 ± 3.52	72 ± 2.64	82 ± 3.76
	$PGPR_2+S_1$	86 ± 1.52	88 ± 3.78	82 ±3.52	90 ± 1.23	78 ± 1.65	84 ± 2.22
	$PGPR_3+S_1$	84 ± 2.64	88 ± 3.21	84 ± 3.33	88 ± 4.21	76 ± 3.42	86 ± 3.51
	$PGPR_1+S_2$	78 ± 4.93	86 ± 6.00	80 ±1.32	84 ± 3.54	74 ± 1.49	82 ± 2.86
	$PGPR_2+S_2$	82 ± 1.52	84 ± 5.52	78 ± 1.04	86 ± 3.00	76 ± 3.51	80 ± 2.55
	$PGPR_3+S_2$	82 ± 2.64	86 ± 5.67	82 ± 3.54	88 ± 2.74	78 ± 2.63	83 ± 2.65
Mean germination time (MGT)	Control	3.2 ± 0.608	3.3 ± 0.541	3.0 ± 0.31	3.5 ± 0.37	2.7 ± 0.41	3.1 ± 0.44
	S_1	3.4 ± 0.642	3.6 ± 0.287	3.2 ± 0.34	3.8 ± 0.27	3.1 ± 0.58	3.3 ± 0.24
	S_2	3.8 ± 0.550	3.7 ± 0.971	3.5 ± 0.48	3.9 ± 0.11	2.8 ± 0.33	3.6 ± 0.75
	$PGPR_1$	2.7 ± 0.577	2.7 ± 0.520	2.5 ± 0.32	2.7 ± 0.42	2.3 ± 0.69	2.6 ± 0.55
	$PGPR_2$	2.7 ± 0.608	2.8 ± 0.482	2.7 ± 0.52	2.8 ± 0.64	2.4 ± 0.44	2.7 ± 0.23
	$PGPR_3$	2.8 ± 0.550	2.7 ± 0.193	2.6 ± 0.54	2.7 ± 0.28	2.4 ± 0.53	2.7 ± 0.35
	$PGPR_1+S_1$	3.2 ± 0.513	2.5 ± 0.271	2.4 ± 0.65	2.3 ± 0.43	2.1 ± 0.22	2.4 ± 0.21
	$PGPR_2+S_1$	3.4 ± 0.520	3.5 ± 0.183	3.2 ± 0.98	3.6 ± 0.33	3.1 ± 0.73	3.3 ± 0.37
	$PGPR_3+S_1$	3.3 ± 0.608	3.5 ± 0.318	3.3 ± 0.41	3.5 ± 0.21	3.1 ± 0.20	3.4 ± 0.66
	$PGPR_1+S_2$	3.1 ± 0.642	3.3 ± 0.219	3.1± 0.62	3.3 ± 0.43	2.9 ± 0.33	3.2 ± 0.23
	$PGPR_2+S_2$	3.2 ± 0.651	3.4 ± 0.211	3.2 ± 0.83	3.4 ± 0.72	3.0 ± 0.46	3.2 ±0.54
	$PGPR_3+S_2$	3.2 ± 0.641	3.4 ± 0.190	3.2 ± 0.69	3.6 ± 0.19	3.1 ± 0.32	3.3 ± 0.31

-Mean of three replicates and ± is standard error (*n*=3), -*Pseudomonas putida* ($PGPR_1$), *P. fluorescens* ($PGPR_2$) and *Bacillus subtilis* ($PGPR_3$) , - Salinity level: S_1= 4000 and S_2= 8000 ppm

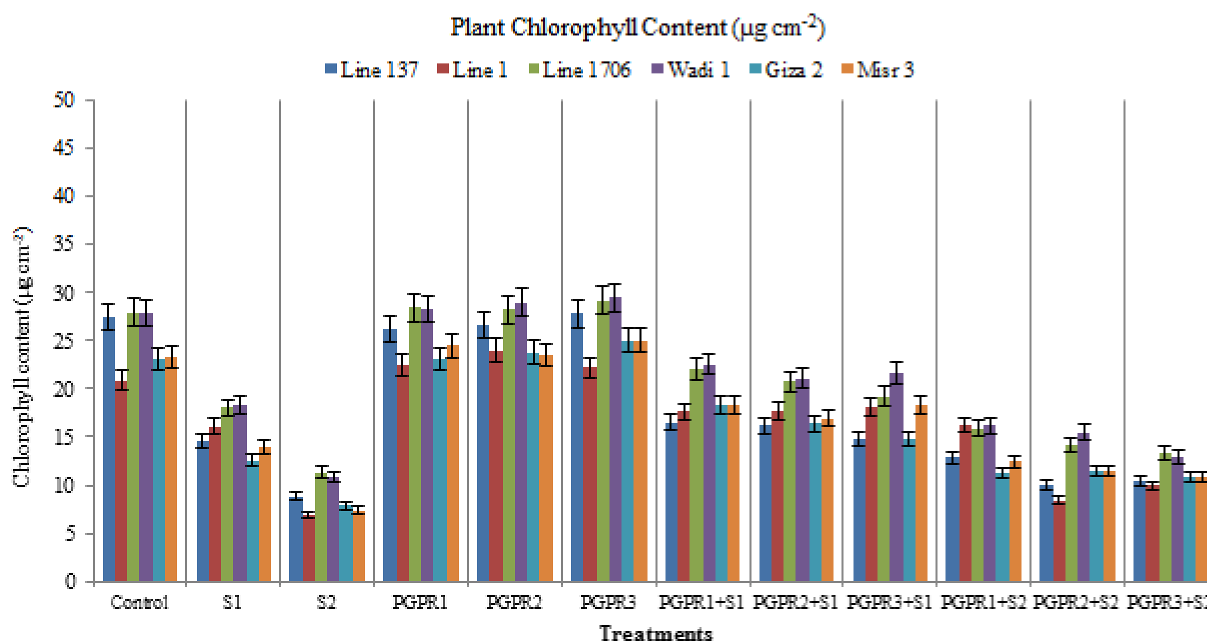

Fig 3. The effect of three PGPR and two levels of salinity on plant chlorophyll content (μg cm^{-2}) for different faba been cultivars -*Pseudomonas putida* ($PGPR_1$), *P. fluorescens* ($PGPR_2$) and *Bacillus subtilis* ($PGPR_3$) - Salinity level: S_1= 4000 and S_2= 8000 ppm.

Table 4. The effect of three isolates of PGPR in the presence of two levels of salinity on some plant growth parameters for six faba bean cultivars.

Parameters	Treatments	Faba bean varieties					
		Line 137	Line 1	Line 1706	Wadi 1	Giza 2	Misr 3
Plant shoot length (cm plant^{-1})	Control	42.66^{a}	43.33^{b}	43.00^{b}	50.00^{a}	40.00^{c}	42.33^{c}
	S_1	23.33^{cd}	27.00^{def}	28.66^{def}	30.66^{cd}	17.66^{e}	18.33^{e}
	S_2	18.00^{d}	21.33^{f}	23.00^{g}	29.66^{d}	17.00^{e}	18.00^{e}
	$PGPR_1$	42.88^{a}	49.33^{ab}	53.33^{a}	51.00^{a}	42.00^{bc}	52.66^{ab}
	$PGPR_2$	43.66^{a}	50.33^{a}	49.66^{a}	55.33^{a}	45.33^{b}	50.00^{b}
	$PGPR_3$	43.75^{a}	51.00^{a}	52.33^{a}	55.00^{a}	51.66^{a}	54.66^{a}
	$PGPR_1+S_1$	33.33^{b}	36.00^{c}	35.66^{c}	38.33^{b}	23.33^{d}	30.00^{d}
	$PGPR_2+S_1$	31.66^{b}	31.33^{cd}	34.33^{c}	34.66^{bc}	20.66^{de}	26.66^{d}
	$PGPR_3+S_1$	28.33^{bc}	30.00^{cde}	33.33^{cd}	31.33^{cd}	20.00^{de}	21.33^{e}
	$PGPR_1+S_2$	21.33^{d}	25.33^{def}	26.66^{efg}	31.33^{cd}	18.00^{e}	19.66^{e}
	$PGPR_2+S_2$	20.66^{d}	24.00^{ef}	25.66^{fg}	27.66^{d}	20.00^{de}	20.00^{e}
	$PGPR_3+S_2$	23.00^{cd}	30.00^{cde}	31.00^{cde}	36.66^{b}	20.00^{de}	21.33^{e}
	LSD 0.05	5.438	6.758	5.284	5.216	3.790	3.789
Plant shoot fresh weight (g plant^{-1})	Control	19.15^{ab}	15.06^{ab}	15.26^{b}	20.48^{a}	15.65^{c}	15.41^{bc}
	S_1	10.50^{de}	10.03^{d}	10.11^{d}	10.31^{bcd}	05.46^{e}	10.05^{f}
	S_2	09.15^{e}	10.01^{d}	10.36^{e}	10.28^{d}	05.35^{e}	10.01^{f}
	$PGPR_1$	19.50^{b}	15.43^{a}	20.13^{a}	21.45^{a}	15.45^{b}	20.10^{ab}
	$PGPR_2$	21.00^{a}	15.45^{a}	20.25^{a}	22.43^{a}	15.46^{b}	20.20^{ab}
	$PGPR_3$	19.38^{ab}	15.36^{a}	20.30^{a}	21.33^{a}	20.48^{a}	20.33^{a}
	$PGPR_1+S_1$	10.43^{c}	10.23^{bcd}	15.01^{c}	15.23^{b}	10.20^{de}	15.08^{cd}
	$PGPR_2+S_1$	10.40^{c}	10.43^{bcd}	10.32^{c}	10.43^{bcd}	05.45^{e}	10.41^{de}
	$PGPR_3+S_1$	10.26^{cd}	10.45^{bc}	15.01^{c}	10.41^{bcd}	05.43^{e}	10.16^{ef}
	$PGPR_1+S_2$	10.06^{de}	10.16^{cd}	10.21^{d}	10.45^{bcd}	10.10^{de}	10.10^{ef}
	$PGPR_2+S_2$	10.03^{de}	10.03^{d}	10.10^{de}	10.35^{cd}	05.43^{de}	10.11^{ef}
	$PGPR_3+S_2$	10.83^{e}	10.33^{bcd}	15.10^{bc}	15.11^{bc}	10.28^{cd}	10.03^{f}
	LSD 0.05	2.485	3.930	2.365	3.740	3.170	3.345
Plant leave area (cm^2)	Control	10.00^{c}	10.83^{b}	11.70^{bc}	10.91^{d}	11.41^{c}	10.75^{c}
	S_1	08.75^{cd}	07.11^{cde}	07.41^{e}	07.08^{ef}	06.75^{e}	7.916^{e}
	S_2	05.83^{ef}	06.66^{de}	05.75^{e}	06.16^{ef}	04.50^{g}	5.833^{f}
	$PGPR_1$	16.20^{ab}	15.25^{a}	13.50^{b}	14.41^{bc}	13.91^{b}	14.50^{b}
	$PGPR_2$	14.80^{b}	15.08^{a}	16.60^{a}	17.66^{a}	15.83^{a}	14.00^{b}
	$PGPR_3$	16.90^{a}	16.25^{a}	16.50^{a}	15.58^{b}	16.00^{a}	16.33^{a}
	$PGPR_1+S_1$	07.66^{de}	08.91^{c}	12.10^{bc}	12.91^{cd}	08.75^{d}	10.58^{cd}
	$PGPR_2+S_1$	05.66^{f}	07.66^{cd}	11.00^{cd}	11.95^{d}	06.58^{e}	09.00^{de}
	$PGPR_3+S_1$	07.41^{def}	07.75^{cd}	09.41^{d}	11.58^{d}	06.25^{e}	07.75^{e}
	$PGPR_1+S_2$	07.91^{d}	07.33^{cde}	06.25^{e}	06.08^{f}	05.00^{fg}	05.00^{f}
	$PGPR_2+S_2$	07.58^{def}	05.66^{e}	06.08^{e}	08.16^{e}	04.33^{g}	4.333^{f}
	$PGPR_3+S_2$	07.91^{d}	07.50^{cd}	06.75^{e}	08.18^{e}	05.83^{ef}	5.833^{f}
	LSD 0.05	1.928	1.825	1.834	2.039	0.972	1.645

-Means in each column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Duncan's multiplerange test. - Values are the means of three replications. - *Pseudomonas putida* ($PGPR_1$), *P. fluorescens* ($PGPR_2$) and *Bacillus subtilis* ($PGPR_3$) - Salinity level: S_1= 4000 and S_2= 8000 ppm.

Table 5. Polymorphism detected by using the 9 selected ISSR primers.

Primer code	Primer sequence	Total amplified fragments	No. of polymorphic fragments	Polymorphism No. (%)	No. of Unique fragments
INC1	$(AG)_8YC$	10.0	5.00	50.0 %	5.00
INC2	$(AG)_8YG$	4.00	1.00	25.0%	3.00
INC3	$(AC)_8YT$	6.00	3.00	50.0%	3.00
INC4	$(Ac)_8YG$	12.0	1.00	8.40%	11.0
INC5	$(GT)_8YG$	6.00	3.00	50.0%	3.00
INC6	$CGC(GATA)_4$	11.0	10.0	90.9%	1.00
INC7	$GAC(GATA)_4$	12.0	6.00	50.0%	6.00
INC8	$(AGAC)_4GC$	13.0	7.00	53.8%	6.00
INC9	$(GATA)_4GC$	17.0	12.0	70.5%	5.00
Total		91.0	48.0	448.6	43.0
Average		10.11	5.33	49.84%	4.77

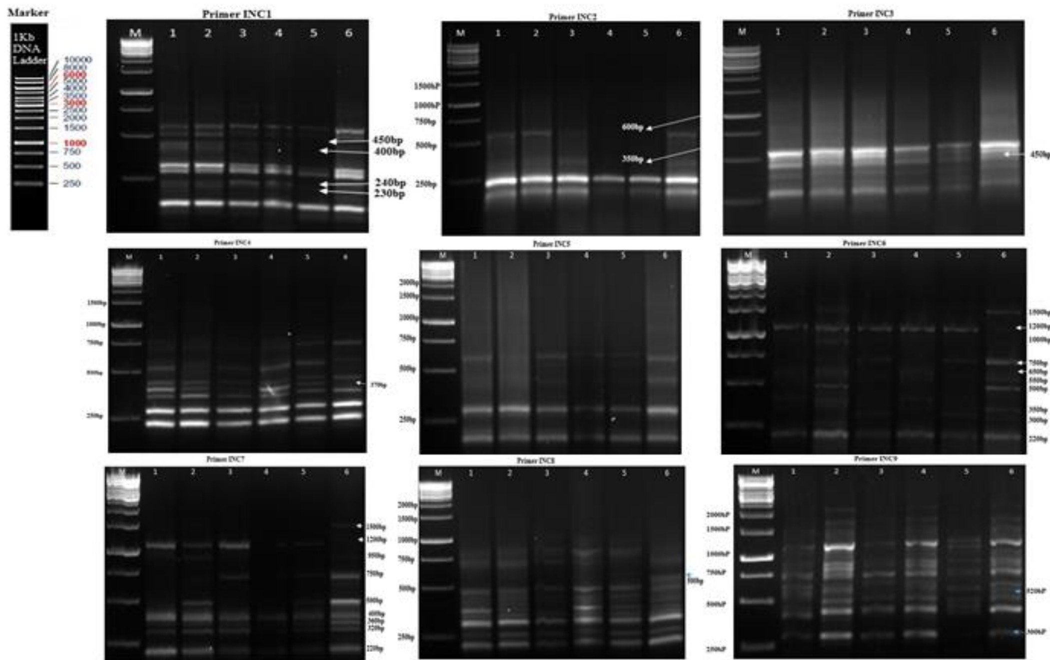

Fig 4. DNA banding patterns generated by different ISSR-PCR primers. M; 1Kb DNA Ladder 250 to 10000. No 1-6 is the cultivars of faba bean as in table 1. 1-Line 137, 2- Line 1, 3- Line 1706, 4-Wadi 1, 5- Giza2 and 6-Misr3.

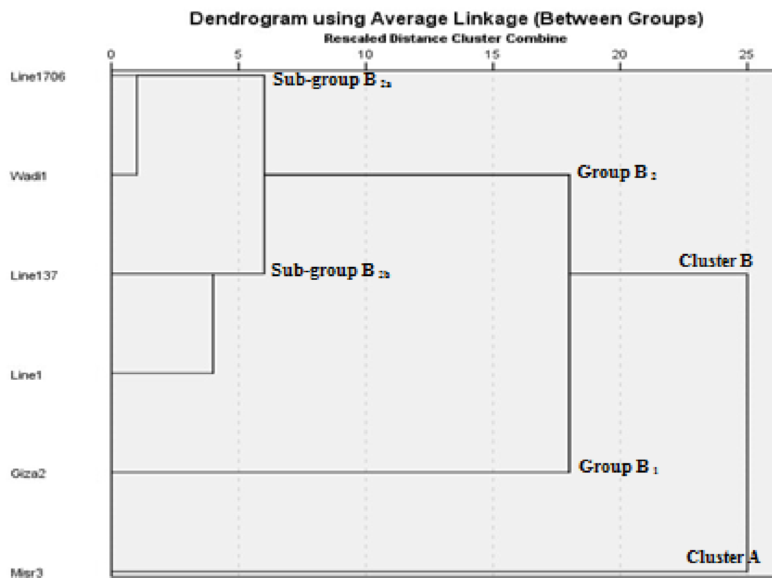

Fig 5. UPGMA dendrogram based on the similarity coefficient showing the clustering pattern among the 6 cultivars of faba bean.

an amplified fragment of 240 bp in all cultivars except cvs. Giza2 and Misr3. This primer also produced an amplified fragments of 450, 400, 230 bp in salt and moderate cultivars only (Fig. 4). The results of ISSR analysis using INC2 and INC4 are illustrated in Fig. 4. These primers produced two amplified DNA fragment of 350 and 370 bp in all cultivars except cvs. Giza 2 and Misr 3, respectively. From the ISSR profiles generated by primer INC6, bands with molecular weight 1200 and 650 bp were absent in sensitive cv. Misr 3 and Giza 2, respectively, in all cultivars. The band with molecular size of 750 bp was presented in salt sensitive cultivars only. Fig. 4 represented the amplified fragment pattern of primer INC7, one negative marker at 1500 bp and one positive marker at 1200 bp were recorded only in cv. Misr3. Using primer INC8, an amplified fragment of 500 bp was generated only in salt sensitive cv. Misr 3 and absent in all other cultivars. Primer INC9 indicted one band at 520 bp while the other band at 300 bp was absent and observed only in cv. Misr 3.

The data on the presence or absence of bands from particular primers for all cultivars was used to similarity based analysis, by which the Jacquard's similarity coefficient was calculated. The genetic similarity coefficient among the faba bean cultivars varied from 0.771 to 0.879 with average genetic similarity of 0.825 for data generated by ISSR markers (Table 7). The highest value (0.879) of genetic similarity were observed between cv. Wadi 1 and both of

Table 6. Number of positive and negative markers for faba bean based on ISSR analysis.

Primer	Number of Markers (bands)	
	Positive	Negative
NIC1	3	1
NIC2	1	0
NIC3	0	0
NIC4	1	0
NIC5	0	0
NIC6	2	1
NIC7	1	1
NIC8	0	1
NIC9	1	1
Total	9	5

Table 7. Similarity coefficient among the 6 cultivars of *Vicia faba* obtained from ISSR markers.

Case	Matrix File Input					
	Line137	Line1	Line1706	Wadi1	Giza2	Misr3
Line137	1.000					
Line1	0.870	1.000				
Line1706	0.842	0.857	1.000			
Wadi1	0.879	0.877	0.879	1.000		
Giza2	0.810	0.814	0.859	0.851	1.000	
Misr3	0.771	0.831	0.859	0.824	0.775	1.000

Line137 and Line1706, while the lowest value (0.771) recorded between cv. Line137 and Misr3. Genetic cluster analysis was conducted using the unweighted pair group methods, then we constructed a genetic relationship dendrogram depends on ISSR analysis. As Fig. 5 shows, the 6 faba bean cultivars have grouped into two major clusters. The first cluster (A) includes only the cv. Misr3, while the other cultivars are predominantly grouped in the second cluster (B), which is comprised of two different subgroups (B_1 and B_2). Group B_2, subdivided into two groups (B_2.a and B_2.b). The cultivars line1706 and Wadi were included in the subgroup B_2.a, while Line137 and Line1 were included in subgroup B_2.b. On the other hand, only cv. Giza2 was included in group B_1.

Discussion

The growth performances of six varieties from *Vicia faba* plants were estimated by growth parameters in the presences or absence three isolates from PGPR and two level of salinity stress. Salinity treatments caused a statistically significant decrease in all growth parameters and plant chlorophyll content, compared to control. Similar reduction in growth performance were found in some plants under saline conditions (Ates and Tekeli, 2007; Azooz, 2009; Ekmekçi and Karaman, 2012; Kaya et al., 2013). This might be attributed to the toxic effect of salinity or increased crucial osmotic pressure, at which the faba bean plants would not be able to absorb water due to osmotic effect and decrease in some physiological activities. The low reduction in growth parameters in the present of salinity stress was found in the treatment with the three isolates form PGPR that may be due to the ability of PGPR to limit Na^+ and Cl^- transport into the shoots. Golpayegani and Tilebeni (2011) observed that inoculation of basil with *Pseudomonas* sp. and *Bacillus lentus* alleviated the salinity effects on growth, photosynthesis, mineral content, and antioxidant enzymes. Dardanelli et al. (2008) observed that PGPR promoted root branching in bean seedling roots and increased secretion of flavonoids and lipochitooligosaccharides. Root-colonizing bacteria which produce auxin under saline condition may supply additional auxin into the rhizosphere, which could help to maintain root growth under stress, and also can contribute to maintaining leaf growth (Albacete et al. 2008). Similar results were obtained by Abbaspoor et al. (2009) who reported increased plant growth of wheat by inoculation with *P. fluorescens* and *P. putida*. Soluble proteins and proline have been shown involved in osmotic regulation in plant, playing an important role in tolerance of plant to salinity stress (Bartels and Sunkar, 2005), and may use as a protective strategy to alleviate Na^+ toxicity (Chen et al., 2002). In the current study, salinity stress caused a significant decrease in soluble proteins, while proline was increased in plants compared to un-salinized control plants. The reduction of protein was previously recorded by Bassuony et al. (2008) and Sadak et al. (2010). They concluded that, the reduction of protein under salinity stress was suppressed by the accumulation of total amino N and proline. The accumulation of proline and amino acids in the cytoplasm plays an important role in the osmotic balance of plants and are good indicators of salinity tolerance (Azooz, 2002; Azooz et al., 2004; Ramezani et al., 2011). These conclusions are confirmed with the results of this study. The increased proline content in faba bean varieties suggests an excellent mechanism to decrease the osmotic potential in this plant. This supports the presumption that proline accumulation is a part of physiological response of plant to intense stress (Ain-Lhout et al., 2001; Rabie and Almadini, 2005).

The 9 selected primers showed high polymorphic banding profile (Table 5) and each of these primers produced fingerprint profiles unique to each of the cultivars; therefore, each primer can be used separately to identify these cultivars. 6 out of the 9 primers [(GATA)4GC, (AGAC)4GC, GAC(GATA)4, (AC)8YC, CGC(GATA)4 and (AG)8YC] generated clear multiplex banding profiles. This was in concordance with previous studies on *Ficus carica* (Gyana and Subhashree 2009) and faba bean (Maryam et al., 2015), where their results showed that the most of the primer based on GA/AG and GT/TG dinucleotide core or dinucleotide motifs (GA)n, (CT)n and (AG)n repeat generated good banding profiles and high level or polymorphism, respectively. These results were explained by Carvalho et al. (2009), who reported that dinucleotide primers were more suitable for amplifying ISSRs and (GA) dinucleotide repeats are most abundant in plant species. Our study showed a low polymorphism level (47.25%). This low or moderate

percentage of polymorphism was also reported in some studies (Sajad et al., 2012; Gong et al., 2011; Wang et al., 2012; Dagnew et al., 2014). The weakness of polymorphism may be probably due to an inherently narrow genetic base and the outcrossing made of pollination.

Among the 48 polymorphism bands, only 14 were found to be useful as positive or negative markers related to salt stress (Table 6). These 14 bands were generated only by primers INC1, INC2, INC4, INC6, INC7, INC8 and INC9, while the rest of primers , INC3 and INC5 were not be used as markers. Primers INC2 and INC4 generated positive marker and only primer INC8 generated negative marker, while primers INC1, INC6, INC7 and INC9 generated both of positive and negative markers (Table 6). A positive marker is a band generated in the salt tolerant cultivars Wadi 1, Line1706, Line137 and Line1, while a negative marker is a band generated in salt sensitive cultivars Giza2 and Misr3. These cultivars, which showed salt tolerance, could be novel to accelerate plant breeding in faba bean. These cultivars can be used as promising lines with more confidence based on their genetic merits rather than phenotypic basis. These results were in agreement with (Reddy et al., 2009; El-Nahas et al., 2011; Rasha, 2013) which demonstrated the effectiveness of ISSR-PCR to enhance the identification of tolerant to environmental stress in different crops. Genetic similarity at ISSR levels in the present study was succeeded to detect the genetic relationship among Faba bean cultivars (Table 7). The highest similarity value were recorded among four cultivars cv. Wadi 1, Line137, Line1 and Line1706 in combinations, which indicated that these four genotypes were closely related to each other. This was reflected by their response to salt stress, while the lowest similarity value were recorded between these cultivars and Giza2 or Misr3, which indicated these cultivars are genetically distant genotype in their salt stress. The grouping of Wadi 1, Line 1706, Line 1 and Line137 in the same group (B_2) shows the greater genetic similarity among these genotypes. It appears from higher genetic similarity observed among these genotypes that it may not generate significant amount of heterosis and not useful in transgressive breeding. The distant grouping of Giza 2 and Misr 3 with Wadi 1, Line 1706, Line 137 and Line 1 offers a possibility of utilizing them in genetic improvement faba bean genotypes in Egypt. This conclusion was supported by Fabio et al. (2010), which indicated that the genetic variability was not expected to be high for faba bean local populations, since they are partially cross pollinated and are heterogeneous mixtures of inbred and hybrids. Based on this result, it is important to assess the validity of molecular markers to select the best salt stress genotypes, testing enough number of genotypes with different genetic background. This study proved the usefulness of unravelling the genetic relationships among closely related genotypes. This comment was also supported by (Caliskan et al., 2012) which concluded that in the future germplasm collection should avoid duplication in the present collection of germplasm.

Materials and Methods

Plant material

Out of 12 faba bean bred by Department of Legume Research, Agriculture Research Center, Cairo, Egypt only 6 faba bean genotypes (Table 1) were selected to use in this investigation with co-operation with Biological Science Department, Faculty of Science, Jeddah University, Saudi Arabia. Screening processes using morphological, biochemical and molecular markers either were conducted in Biological Science laboratory and greenhouse, respectively, during season 2013-2014.

Mass culturing of plant growth promoting rhizobacteria (PGPR)

The isolates of PGPR were supplied by microbiology Lab of the Faculty of Sciences, Jeddah University, Saudi Arabia. PGPR included *Pseudomonas putida* ($PGPR_1$), *P. fluorescens* ($PGPR_2$) and *Bacillus subtilis* ($PGPR_3$). They were cultured on Broth nutrient. For making the stock solution, their culture was mixed in 100 ml of 1% glucose solution to have the concentration of 1×10^7 CFU/ml of each PGPR.

Preparation of seawater dilution

The required quantity of seawater for the entire experiment was collected from Red Sea Beach, Jeddah City. The seawater salinity level was 34000 ppm. Two different salt concentration (S_1 and S_2) in ppm were prepared to use in this experiment by diluting sea water to 11.7% and 23.5% by adding distilled water to obtain S_1= 4000 and S_2= 8000 ppm, respectively.

Effect of PGPR on faba bean germination rate and seedling growth (in-vitro)

To study the effect of PGPR on plant germination rate in salinity stress conditions 6 cultivars of faba bean plants were used. About 50 seeds from each 6 cultivars of faba bean were sterilized by soaking in 2% sodium hypochlorite for 5 min and then they were washed by sterile distilled water for 5 times. Twenty four treatments were replicated three times as following: (1) Seeds were incubated in 100 ml of 1% glucose solution as a suspension of the three PGPR isolates (*Pseudomonas putida*, *P. fluorescens* and *Bacillus subtilis*) separately with rate of 1×10^7 CFU/ml at room temperature for 4 h. (2) Seeds were incubated with 1% glucose solution free from bacterial cells as control. After incubation period, the soaked seeds were placed in sterilized pot containing wet peat-moos. (3) Seeds were treated with two seawater salt solutions (4000 and 8000 ppm) plus control (tap water), separately by irrigating treated pots twice in a week with 300 ml sea salt solution pot^{-1}. (4) Seeds were also treated with both each of the three PGPR in the presence of both salt solutions. Pots were incubated in growth room at 28±2°C for 3 days to calculate the final germination percent (FGP) (ISTA, 1993; 1999) based on the following equation:

$$FGP = \frac{\text{number of germinating seeds}}{\text{Total number of seeds}} \times 100$$

Mean germination time (MGT) was calculated according to the following equation (Moradi et al., 2008):

$$MGT = \sum Dn / \sum n$$

Where, "*n*" is the number of seeds germinated on day "*D*", and "*D*" is the number of days counted from the beginning of germination.

Effect of PGPR on faba bean growth and biochemical traits in greenhouse

In a greenhouse the bean seeds were planted in plastic pots (14 cm diameter and 12 cm depth) containing 40% Sand: 30% clay: 30% peat-moos in completely randomized design (CRD) at temperature 30±2°C and 60% relative humidity for

90 days. The plants were grown with non-saline irrigation water for three weeks in order to ensure proper establishment. About 500 mg pots^{-1} of NPK fertilizer was applied fortnightly. After that, twenty four treatments were replicated three times as described above in laboratory experiment. At the end of the experiment, plants were harvested to determine some parameter such as shoot fresh weight (g/plant), shoot length (cm), plant leave area (cm^2), plant proline content (µg g^{-1}FW), protein content (mg g^{-1}FW) and the chlorophyll content in leaves (µg cm^{-2}) which measured by Chlorophyll Content Meter (model CL-01 Co. Hana Tech Instruments).

Analytical method for soluble protein and free proline

Soluble proteins content (mg g^{-1} FW) was determined spectrophotometrically according to Bradford (1976). The sample extract (0.5 ml) was prepared by homogenized 5g of plant leaves with 0.5 ml distilled water and 3 ml of Coomassie Bio Rad dye. Absorbance was read at 595 nm after five minutes. The free proline content was estimated using the acid ninhydrin method as described by Bates et al. (1973). Five grams of plant leaves were grounded in a mortar and pestle with 3% (w/v) sulfosalicylic acid aqueous solutions and the homogenate was filtered through Whatman No. 1 filter paper, then 2 ml of filtered extract was taken for the analysis to which 2 ml acid ninhydrin and 2 ml glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h and the reaction was finished in an ice bath. Four ml of toluene was added to the reaction mixture and the organic phase was extracted, in read at 520 nm using toluene as blank by UV-visible spectrophotometer (Thermo Electron, Model Bio Mate 3, Massachusetts, USA). Proline concentration was determined using calibration curve and expressed as ug g^{-1}FW.

Molecular analysis

Extraction and purification of genomic DNA

The DNA was extracted, from 0.2 g of fresh young leaf tissues of plants chosen randomly, by Qiagen DNeasy kit (Qiagen Santa Clara, CA). DNA concentration was determined by diluting the DNA 1:5 in dH2O. The DNA samples were electrophoresed in 0.7% agarose gel against 10 µg of a DNA size marker (Lambda DNA digested with *Hind III* and Phi x 174 DNA digested with *Hae III).* This marker covers a range of DNA fragments size between 23130 bp and 310bp, and a range of concentration between 95 ng and 11 ng. Thus, estimation of the DNA concentration in a given sample was achieved by visually comparing the degree of fluorescence of the unknown DNA band with the different bands in the DNA size marker.

ISSR analysis

PCR was performed in 25 µl reaction volume containing 2X ready mix (EmeraldAmp Max PCR master mix) 25 pM oligonucleotide primer and 50 ng genomic DNA. A set of 9 ISSR primers (Table 2), synthesized by Bioron Corporation, Germany, were used in this study. DNA amplification was performed on Eppendorph Master Cycler programmed to 35 cycles using PerkinElmer, Inc. Cetus480 DNT Thermal Cycler (PerkinElmer, Inc. Cetus, Norwalk, Conn, USA) as follows: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation step at 94°C for 1 minute, annealing temperature (Ta) for 1 minute, and an extension step at 72°C for 1 minutes, and final extension step at 72°C for 10 minutes. Amplification products were separated by horizontal gel electrophoresis unit using 1.5 % (w/v) agarose gel on 0.5 × TBE buffers (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) under a constant voltage of 80 V for 2 h, stained with 1 μg ml^{-1} ethidum bromide. Thermo Scientific Gene Ruler 1Kb DNA Lader 250 to 1000 bp was used as DNA marker and applied in the first column of gel followed by the samples that arranged on the gel from left to right as a numeric number (1-2-3-4-5-6). Bands were visualized in UV transilluminator at 300 nm and photographed using gel documentation equipment (Bio Rad). The banding patterns were visualized on transilluminator. The banding patterns were scored as present (1) or absent (0). The similarity of all samples for all scored bands was assessed using Jacquard's similarity coefficient and the matrices generated were analyzed with SPSS version 12 software to evaluate genetic distance.

Data analysis

Data were analyzed using analysis of variance (ANOVA) by using SAS statistical software (SAS Institute, Cary, NC, USA, 1998). The significance of differences within treatments was separated by Least Significant Difference test at 5%.

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