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Effect of Rhizobium spp. Activated by Colchicine on Morphology, Anatomical Features and DNA Content of Soybean Plant (*Glycine Max*).

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ABSTRACT

A significant legume crop known for its high levels of protein and edible oil is the soybean. High levels of nitrogen are needed by soybean plants, and this nitrogen is primarily supplied by biological nitrogen fixation. A field test was performed by using soybean (Glycine max) Giza 82 cultivar.Growing soybean with or without Rhizobium spp. and Colchicine compared to non-inoculated plants, led to raise in some morphological features similar to this, infected plants displayed increased enzyme activity. Such as catalase, polyphenol, peroxidase and ascorbate oxidase,as compared to soybean plants that weren't inoculated . Colchicine concentration was the most crucial source of diversity across all traits. Significant responses were detected for various morphological parameters, anatomical features of root and leaf, and DNA content in the genotype used.High polymorphic bands were found in ISSR tested primers, and fingerprinting revealed 125 distinct DNA fragments in all has 105 fragments on average each primer. There were between one and three polymorphic bands per primer, with an average of 0.75.

KEYWORDS: *Glycine max*;*Rhizobium spp*.Colchicine, ISSR, Molecular markers

1. INTRODUCTION

Leguminous pulse crop Glycine max (L.) Merr. (Fabaceae), also known as soybean, is an important one. This crop has higher concentrations of nutrients that are good for your health, including as unsaturated fats, vitamins, essential oils, and proteins(Jeong, *et al.,* 2010).Colchicine, a possible antimitotic mutagenic drug that blocks microtubule function in chromosome segregation, was also the subject of research (Manzoor, *et al.*, 2015).Colchicine, one of the mutagenic agents, specifically affects the mitotic and spindle fibres' structure and orientation. When plants are successfully pre-treated with this chemical, such effects lead to subsequent increases in growth traits like the sizes of leaves, fruits, and seeds.In many breeding systems, this form of genetic

alteration has long acquired appeal. The method is still a good option, especially to genetic engineering, because it has attracted a lot of interest from consumers and academics and greatly allays worries about genetic instability and potential cancer-causing activities. Biofertilizers or microbial inoculants are terms used to describe live microorganism (bacteria) preparations used to increase plant growth and agricultural productivity. The first known biofertilizers were Nitrogen from the atmosphere is fixed by Rhizobium species. For more than a century, these organisms have been employed commercially as legume inoculants. (Kannaiyan, 2002). Numerous microbial inoculants or biofertilizers, such as PGPR, vesicular-arbuscular mycorrhizae (VAM), phosphate-solubilizing microorganisms, and nitrogen-fixing bacteria, have been developed as a consequence of research in the subject of biofertilizers. A wide range of plant species, among them rice (Joshi et al., 2000), apples (Goulo and Oliveira, 2001), and strawberries (Arnau et al., 2003), have had their cultivars identified using ISSR analysis. In plant genomes, Multilocus patterns produced by ISSR-PCR are widely distributed, polymorphic, and highly reproducible. (Bornet and Branchard, 2004). The efficiency of PCR-based markers comprising ISSR in plant breeding has been demonstrated by comparisons between them (Adams et al., 2003; Archak et al., 2003; Galvan et al., 2003). According to Zohary (1966), A. macrostachyum is extensively spread around the world, but is primarily found in the Mediterranean and Saharan regions of Arabia. Mir et al. (2021) found that Six bacterial strains that resemble rhizobia were found in the stem and root nodules of various leguminous plants. increased plant height, branch count, total chlorophyll, and nodule size.

2. MATERIALS AND METHODS

2.1. Plant materials

Soybean (*Glycine max* L.) seeds were acquired from the Field Crop Institute, Legumes Department, at the Agricultural Research Centre in Giza, Egypt. The name of the cultivar, its provenance, pedigree, and the weight of 100 seeds were recorded.

2.2. Pre-treatment and germination of seeds

Based on explanation by Mangena et al. (2021), the soybean seeds were originally examined for germination by surface disinfection. The seeds were pre-treated with a concentration of 1.0% colchicine by pre-soaking them for 12 hours after sterilisation. The epicotyl's emergence was regarded as the beginning of germination.

Studied some morphological characters:

Statistical analysis

Five plants were selected to represent each of the three replicates of each treatment. According to Snedecor and Cochran (1989), data were subjected to proper statistical and traditional methods a variance analysis. When P 0.05 was applied, the mean differences were examined using a least significant difference test (L.S.D.).

2.3. Enzyme analysis

Activity of antioxidant enzymes

Fresh leaves (0.5 g) were homogenised in 10 ml of ice-cold, 50 mM phosphate buffer (pH 7.8), 1 mM EDTA, and 2% (w/v), polyvinylpyrrolidone (PVP). The homogenate was filtered through four layers of cheese cloth and centrifuged at 5000 x g for 10 min at 4 oC. Zhang and others (2007). In order to spectrophotometrically assess the activities of peroxidase (POD), polyphenoloxidase (PPO), and catalase (CAT), the supernatant was collected, and ascorbic acid oxidase (AAO). For five minutes, readings from the spectrophotometer were recorded for the enzymes. With the exception of the substrate solution being swapped out for extraction buffer, the reference cuvette for the spectrophotometer always contained the same concentrations of each component as the sample cuvette.

Peroxidase (POD) activity

In the case of Kato and Shimiz (1987), the reaction mixture consisting of 100 mM sodium phosphate buffer (pH 5.8), 7.2 m Mguaiacol, 11.8 mM H2O2, and 100 l enzyme extract was used to assess the activity of POD. H2O2 was used to kick off the reaction, and a change in absorbance at 470 nm was noted. POD activity was measured at 470 min-1 g 1 FW.

Polyphenol oxidase (PPO) activity

Mayer et al. (1966) claimed that PPO's activity had been discovered. A 100 mM concentration of catechol in 200 l was added to the reaction mixture. The change in absorbance was measured at a wavelength of 490 nm, and PPO activity was calculated to be 470 min-1 g 1 FW.

Catalase (CAT) activity

The Aebi (1984) method was implemented to evaluate the activity, in which a reaction mixture containing 0.3 ml of 3% H2O2, 2.5 ml of 0.05 M phosphate buffer (pH 7.0), and 2.5 ml of plant extract serves as a marker for the disappearance of H2O2. The PPO activity unit is 470 min-1 g 1 FW.

Ascorbic acid oxidase (AAO) activity

Ascorbic acid oxidase (AAO) activity has been detected using the approach proposed by Oberbacher and vines (1963). In a nutshell, 8.8 mg of ascorbic acid in 300 ml of phosphate buffer, pH 5.6, and 3.0 ml of the substrate solution had been added to 0.1 ml of the enzyme extract (supernatant). The absorbance change was measured at 470 nm, and the PPO activity was listed as 470 min-1 g 1 FW.

2.4. Bacteria isolation

Rhizobium (Rhizobium spp.) was isolated from soybean plants in the Legumes Department, Field Crop Institute, Agricultural Research Centre, Giza, Egypt, adopting a process specified by He *et al.* (2008).

2.5. Experimental setup.

Peat moss that had been heat sterilized and four replicates of each treatment were used in the experiment, which was conducted in 30 cm plastic pots. On the soybean plant Giza 82 cultivar, the effects of treatments with and without rhizobium inoculation were investigated. The amount of rhizobium in this treated inoculum employed was 1000 mg.

2.6. Anatomical study

At Cairo University's Agric. Bot. Department of the Faculty of Agriculture, microtechnique exercises were conducted. sample of leafs at node No. 5 were taken in addition with root were fixed for at least 48 hours after killing. in a solution containing formaldehyde, acetic acid, and alcohol (F.A.A.) was embedded in paraffin wax after the drying process (Sass, 1951). Sections that were cut on a rotary microtome at a thickness of 15-20 microns were coloured with crystal violet/erythrosine before to mounting in Canada balsam. Slides were examined under a microscope and through a camera.

2.7. DNA extraction and amplification

Total DNA was isolated from fresh leaves using the DNeasy Plant Mini Kit (QIAGEN, Germany) in keeping with the manufacturer's instructions.

2.8. ISSR "Inter Simple Sequence Repeat"

ISSR-PCR Reactions

Table (1) the twelve ISSR primers that were used to find polymorphisms. The amplification reaction, as reported by Ibrahim et al. (2019), was carried out.

2.9.Thermocyling Profile PCR and detection of its Products:

The PCR amplification was done using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) with a 40-cycle capacity after a first denaturation cycle lasting 5 minutes at 94°C. A denaturation stage lasting one minute at 94 °C, an annealing step lasting one minute at 45 °C, and an elongation step lasting one and a half minutes at 72 °C made up each cycle. The primer extension phase was extended in the previous cycle to 7 minutes at 72 degrees Celsius (Hammer et al., 2001).

2.10. Data analysis

All samples were used for the ISSR study, and the final data sets contained both polymorphic and monomorphic bands. Only distinct, unmistakable bands were visually assessed as either present (1) or absent (0) for all samples. Then, a binary statistic matrix was created using PAST software Version 1.91 to correspond with the Euclidean similarity index (Hammer et al., 2001).

Table 1. Selected 12 ISSR primers sequences for cultivar Giza 82 soybean genotype analysis.

Primer Name	Sequence
ISSR-1	5'-AGAGAGAGAGAGAGAGC-3'
ISSR-2	5'-AGAGAGAGAGAGAGAGG-3'
ISSR- 3	5'-ACACACACACACACACT-3'
ISSR- 4	5'-ACACACACACACACACG-3'
ISSR -5	5'-GTGTGTGTGTGTGTGTG-3'
ISSR -6	5'-CGCGATAGATAGATAGATA-3'
ISSR -7	5'-GACGATAGATAGATAGATA-3'
ISSR- 8	5'-AGACAGACAGACAGACGC-3'
ISSR -9	5'-GATAGATAGATAGATAGC-3'
ISSR -10	5'-GACAGACAGACAGACAAT-3'
ISSR- 11	5'-ACACACACACACACACA-3'
ISSR- 12	5'-ACACACACACACACACC-3'

3. RESULTS AND DISCUSSION

3.1. Morphological characters

Table (2) give note about some morphological characters of soybean G82 plants treated by *Rhizobium spp*. (1000mg) compared with control.There was significant increasing in shoot length (cm) by34.8%, while root length increased by 182.4%, also leaves numbers increased by 47.9%compared with control. In order to determine combinations of microbes that have an impact on cell division and differentiation, Hungria *et al.* (2013) and Moretti *et al.* (2019) noticed that the effects of Rhizobia and other plant When soybean root length was exposed to growth-promoting rhizobacteria (PGPR) and their metabolites, it increased by 19.7%.

Table 2. Some morphological charactersof soybean G82 plants treated by *Rhizobium spp.* (1000mg) after 3 months of culturing compared to non inoculated

Treatment	Shoot length (cm)	Root length (cm)	Leaves No.
Control	31.6±0.71	4.1±0.29	7.3±0.38
***Rhizobium spp*. (1000mg)**	42.6±1.23	11.6±0.50	10.8±0.11
LSD	3.04	1.24	0.85

After being exposed to six rhizobia-like bacterial strains, Mir *et al.* (2021) found that the ICCV 2 variety of chickpea (*Cicerarietinum* L.) displayed improvements in plant height, number of branches, total chlorophyll, nodule number, nodule weight, shoot weight, root weight, root volume, and root surface area.

They were isolated from nodules in the stem and roots of different leguminous plants.In addition to nitrogen and phytohormones like indole acetic acid, these beneficial bacteria produce exopolysaccharides, siderophores, mineral solubilizations, and antagonistic action against various phytopathogenic fungi. As mentioned by Shome *et al.* (2022), Rhizobium, on the other hand, has a favourable impact on soybean production, growth, and quality, which supports an increase in the availability of soil nutrients. According to Almeida *et al.* (2022), A. brasilense, which is present in the plant and secretes phytohormones such auxins and cytokinins, favours root growth and the presence of R. tropici.

3.2. Enzyme activity

The effects of *rhizobiumspp*. and Colchicine (1000mg) on some enzyme's activity such as catalase, polyphenol, peroxidase and ascorbate oxidase compared with control were cleared in Table (3). The increased percentage 11.3% in Catalase activity was found, while in polyphenol amounted to 11.6%, peroxidase reach to 30.5% and Ascorbate oxidase increased in activity by 34.8% over the control. Generally, Ascorbate oxidase activity showed the greatest variations in enzyme activity between control plants and plants treated with Rhizobium spp. in the study by Bayat *et al.* (2014), rhizobium inoculation and SO2 treatment substantially lowered the negative effects of high SO2

Table 3. Effect of *rhizobium spp.* (1000mg) on enzyme'sactivity.

Treatment	Catalase (Δ 470 min −1 g −1 FW.) Mean±SE	Polyphenol oxidase (Δ 470 min −1 g −1 FW.) Mean±SE	Peroxidase (U/mg F.W) (Δ 470 min −1 g −1 FW.) Mean±SE	Ascorbate oxidase (Δ 470 min −1 g −1 FW.) Mean±SE
Control	35.1±2.67	35.9±2.25	30.2±3.15	31.6±0.71
***Rhizobium spp.* (1000mg)**	39.1±1.85	40.1±1.48	39.4±0.68	42.6±1.23
LSD	6.94	5.75	6.87	3.04

concentration on root development, antioxidant activity, and capacity. According to Kong et al. (2015), CAT activity in the roots of inoculated plants under Cu stress was substantially greater at 3, 13, and 18 dpi, or by 44.8, 28.5, and 31.2%, respectively, than that of non-inoculated plants. Ylmaz and Kulaz (2019) propose that PGPR may enhance the activity of antioxidant enzyme in chickpea, mitigating the adverse effects of ROS. According to Motamedi et al. (2022) and Amine-Khodja et al. (2022), these findings inspire new ideas for biofertilizer preparation that aim to lessen environmental pressures. The resilience of legume plants to drought stress is influenced by rhizobium strain.

3.3. Anatomical study

Table (4) and (5) and Figure (1) show the effect of *Rhizobium spp.* (1000mg) on rootand leaf anatomical characters.

Table 4. Effect of *Rhizobium spp.* (1000mg) on root anatomical characters.

Root anatomical parameters	Control	*Rhizobium spp.* (1000 mg)
Root diameter, µm	319.3	401.6
Cortex thickness,µm	76.1	77.6
Xylem vessel diameter	28.4	43
Pith, µm	85.4	133.4

Regarding the root structure, there is no significant effect on root cortex of control and treated plants but root diameter increased by 25.8% while xylem vessel diameter by 51.4%.

Table 5. Effect of *Rhizobium spp.* (1000 mg) on leaf anatomical characters.

Leaf anatomical parameters	Control	*Rhizobium spp.* (1000 mg)
Upper epidermis thickness, µm	14.1	17.3
Lower epidermis thickness, µm	12.2	8.2
Midrib thickness, µm	99.3	391.8
Mesophyll thickness, µm	57.6	102.9
Vascular bundle diameter	59.2	186.2
xylem vessel diameter	13	26.4

Concerning the leaf structure, leaf midrib thickness increased by 294.5%, mesophyll thickness by 78.6%, vascular bundle diameter by 214.5%, xylemvessel diameterby 103.1%compared with the control.Helaly *et al.* (2009) indicated that biofertilization caused an raised in leaflet thickness at the midrib because the palisade and spongy tissues grew thicker. The vascular bundle's length and width were both enhanced in the midrib. The effects of biofertilizer treatments on the rise in leaflet internal structure, specifically, IAA, GA3, and cytokinins encouraged cell growth and division as well as the production of fresh cells and plant tissues. , may be linked to these hormones. According to El-Shaarawi *et al.* (2011),

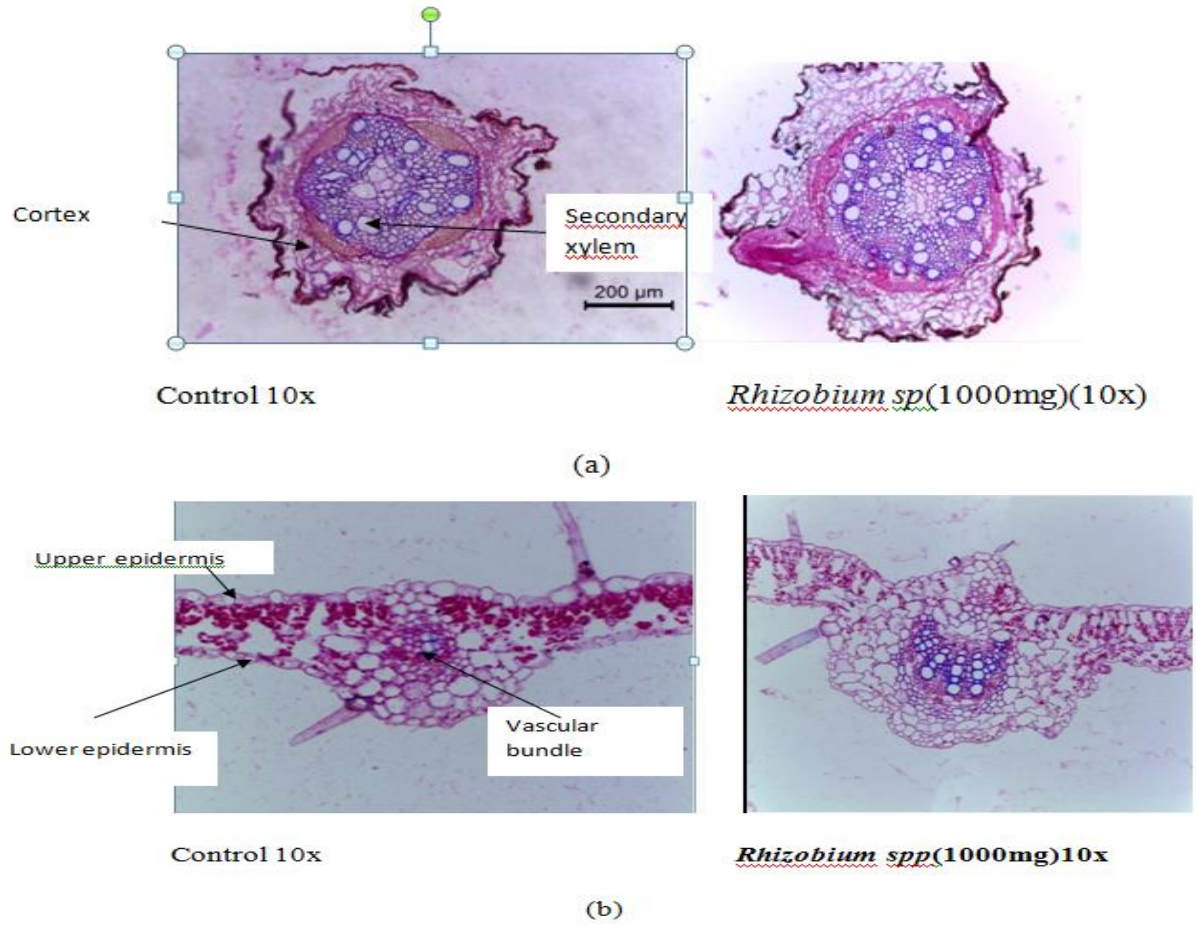

Fig 1. Transverse sections of soybean (a)root and (b)leaf treated with *Rhizobium spp.* 1000mg compared with the control (10X).

inoculating soybean plants with B. japonicum resulted in a modest increase in the thickness of the leaflet blade tissues, but there were no discernible variations in the cortical thickness between inoculated and uninoculated roots. Vaccinated roots.An infected plant exhibits mesophyll consisting of A spongy parenchyma with two layers of palisade cells and a lot of intercellular space gaps, which Paradiso *et al.* (2017) theorized explains the increase in mesophyll. On the adaxial epidermis, stomata were common; on the abaxial surface, they were uncommon. In opinion of Livingston *et al.* (2019), nodules have a more durable vascular structure to facilitate nitrogen fixation.

Molecular markers by using ISSR analysis:

In order to identify molecular markers for differentiation, twelve oligonucleotide primers were utilised to create ISSR-PCR fingerprints of the Giza 82 soybean cultivar. The number and size of the amplified products varied greatly depending on the primer used (table 6). Results of the examined soybean cultivar's ISSR-PCR are provided. The twelve High polymorphic bands have been identified using ISSR tested primers., according to the results presented in Table 6 at this time. The results of the fingerprinting process revealed 125 distinct DNA fragments, ranging about 10.4 pieces per primer. With an average of 0.75 polymorphic bands/primer, the number of polymorphic bands varied between 1 and 3. The tested primers generated 9 polymorphic amplicons in total, which equals an 84.28% polymorphism level (Table 6 and Figure 2) .Between 7.69% and 30.0% of the bands were polymorphic, depending on the ISSR-2 and ISSR10 primers. The amplification with the last 10 ISSR primers produced highly instructive patterns, as seen in Fig. (2). In Brassica and Arabidopsis thaliana, Bornet and Branchard (2004)) confirmed that ISSR primers generated 13 to 26 markers.

Table 6. Polymorphic bands of Soybean generated by ISSR-PCR analysis.

Primer Code	Size Range (bp)		Monomorphic bands	Polymorphic With unique	Total number of bands	Polymorphism (%)
ISSR-1	900	100	8	0	8	0.0
ISSR-2	1500	150	12	1	13	7.69
ISSR- 3	1000	140	9	3	12	25.0
ISSR- 4	1300	150	13	0	13	0.0
ISSR -5	700	150	7	0	7	0.0
ISSR -6	900	150	9	0	9	0.0
ISSR -7	900	200	7	1	8	12.5
ISSR- 8	1100	150	12	0	12	0.0
ISSR -9	1100	160	10	1	11	9.09
ISSR -10	1000	200	7	3	10	30.0
ISSR- 11	1100	200	11	0	11	0.0
ISSR- 12	900	200	11	0	11	0.0
Total	12400	1950	116	9	125	84.28**%**
Average		162.5	9.66	0.75	10.4	7.02

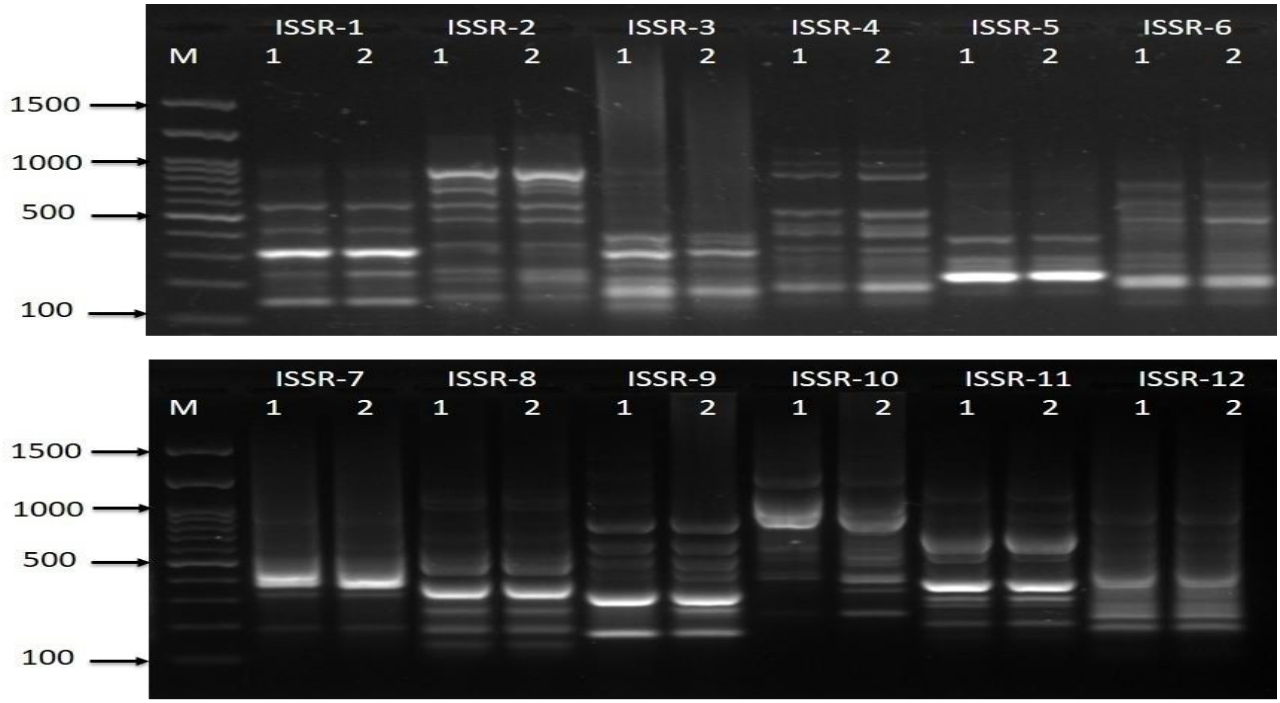

Figure 2. The banding profile of Inter simple sequence repeat (ISSR) screened under study of Giza 82 soybean cultivar, DNA marker 1 kb.

4. CONCLUSION

This study shown that inoculating the Giza 82 soybean (*Glycine max*) cultivar with Rhizobium species and colchicine raised plant shoot, root, and leaf lengths as well as histological characteristics.

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المستخلص العربي

تاثير الريزوبيم المنشطة بالكولوشيسين علي الخصائص المورفولوجية والتشريحية والطرز الجيني علي نبات فول الصويا(*Glycine Max*)

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[١]قسم النبات الزراعي (الوراثة) ، كلية الزراعة ، جامعة الأزهر بالقاهرة ١١٨٨٤، مصر
[٢]قسم النبات الزراعي، كليةالزراعة، جامعة القاهرة، الجيزة، مصر

يُعد فول الصويا من اهم المحاصيل البقولية التي تشتهر باحتوائها على البروتين وزيت الطعام. وتتطلب نبات فول الصويا كمية كبيرة من النتروجين والذي يتم الحصول عليه من التثبيت الحيوي للنتروجين. وقد أجريت التجربة الحقلية باستخدام أواني بلاستيكية ٣٠ سم تحتوي على بيتموس معقم وتم زراعة صنف جيزة ٨٢ من فول الصويا. وقد لوحظ أن طول الساق والجذر وعدد الأوراق للنباتات المعاملة بالريزوبيم والكولوشيسين أعطت نتائج أكبر من الغير معاملة. وأيضا زيادة النشاط الانزيمي لانزيم الكتاليز والبولي فينول اوكسيدز والبروكسيدز واسكوربك اوكسيدز مقارنة بالغير معاملة. وكان تركيز الكولشيسين أهم مصادر التباين لجميع الصفات. لوحظ أيضًا تأثير زيادة معنوية على الصفات المورفولوجية والتشريحية للجذر والورقة ومحتوى الحمض النووي في التركيب الجيني.

الكلمات المفتاحية: فول الصويا، الريزوبيم، الكولشيسين،ISSR، المعلمات الجزيئية.