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Effect of pesticides on Exopolysaccharide (EPS) production, antibiotic sensitivity and phosphate solubilization by Rhizobial isolates from *Sesbania bispinosa* in Bangladesh

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***Rhizobium spp*. retains a symbiotic relationship with leguminous plants including *Sesbania bispinosa* by fixing N_2 through nodule formation. Several researches suggest that Exopolysaccharides (EPSs) are required for nodule formation. Rhizobial growth parameters as well as the EPS production are affected by the presence of pesticides. The present investigation was performed using three different pesticides which were Imitaf 20SL (Insecticide), Tafgor 40EC (Insecticide) and Tilt 250EC (Fungicide). Production of EPS was exceptionally increasing with the escalating concentrations of pesticides. The effects of pesticides were also observed on the antibiotic resistance of these organisms. Some gained resistance against Kanamycin while some got more sensitive than before. Detection of *nod*C gene and *nif*H gene ensured the fact that they are the residents of rhizobia bacteria. This study uncovers the fact that extensive use of pesticides may cause an unfavourable environment for survival of rhizobia and a decrease in EPS production resulting in poor N_2 fixation and thus affecting the whole agricultural economy of a country.**

Key words: Pesticides, Rhizobia, exopolysaccharide, symbiosis, *Sesbania bispinosa*, antibiotics.

INTRODUCTION

Sesbania is a well-known plant in Bangladesh for its varied uses and a member of the 'Fabaceae' family (Sarwar et al., 2017). Most of the species of this genus can nodulate in a symbiotic manner and take part in the nitrogen fixation process. Among them, *Sesbania bispinosa* (locally known as 'Dhaincha') can grow at a fast rate in the swampy and saline environment compared to other legumes, possessing high ability to fix a large amount of N_2 greater yield (Ladha et al., 1988; Ventura and Watanabe, 1993). Since Bangladesh is a

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ABBREVIATIONS: EPS, Exopolysaccharide; **SL,** soluble liquid; **EC,** emulsifiable concentrate; **nod,** nodulation; **nif,** nitrogen fixation.

riverine country and has suitable weather to grow dhaincha, farmers have been cultivating it for a long time to increase the organic content of the soil (Sarwar et al., 2017). In addition to being used as green manure, dhaincha can be used as food for animals, a raw material for pulp production, wood for fuel (Shahjalal and Topps, 2000; Sarkar et al., 2017). So in the context of Bangladesh's climate, dhaincha has become more important to farmers to get its versatile advantages.

In case of *Sesbania bispinosa* and rhizobial symbiotic relationship, a variety of rhizobia induces nodulation represented by the genera such as *Rhizobium, Sinorhizobium, Agrobacterium, Mesorhizobium* and *Bradyrhizobium* (Dreyfus et al., 1988; Ribeiro and Burkert, 2016). When rhizobia invade root cells of the host plant, they form nodules where they fix atmospheric nitrogen (Lepek and D'Antuono, 2005). The rhizobia legume symbiosis starts with the secretion of flavonoids (chemical attractant for rhizobia) which are excreted by the host plant root cells (Brencic and Winans, 2005). While rhizobia start synthesizing and secreting Nod factors, the root cells of the host plant initiate root curling. After trapping into the curled root hairs, rhizobia begin invading these nodules through EPS production (Philip-Hollingsworth et al., 1989; González et al., 1996; Mukherjee et al., 2011). So, EPSs are thought to play a crucial role in the invasion process. Mutants lacking the ability to produce EPSs are unable to invade the root nodules although they could induce nodule formation (González et al., 1996; Janczarek et al., 2014).

Generally, exopolysaccharides impart mucoid appearance to bacterial colonies grown on laboratory agar media (Nwodo et al., 2012). Exopolysaccharides are high molecular weight polymers which are made up of homopolysaccharides or heteropolysaccharides units. The composition of EPSs produced by rhizobial strains includes glucose, galactose, mannose, rhamnose, glucuronic acid and galacturonic acid (Gupta and Diwan, 2017).

It has been observed that EPS production and the whole symbiotic relationship gets disturbed by the harmful influence of pesticides (Ahemad and Khan, 2013). Pesticides keep the plants safe by controlling pests and preventing diseases caused by the pests but in some cases, they cause great damage to the surrounding microflora, plant growth and other factors (Gupta et al., 2014). So the present study focuses on the impact of pesticides on rhizobia especially on their EPS production along with other factors.

MATERIALS AND METHODS

Sample collection

Root samples from *S. bispinosa* were collected from different regions of Bangladesh. The samples contained both nodules and rhizospheric soil. These were further processed for isolating rhizobial strains and tagged as R1 to R44. Forty-two isolates (except R33 and R34) were studied for further biochemical tests.

Isolation and identification of rhizobia

At first, the nodules were washed thoroughly and separated from the roots by using sterile forceps. The undamaged nodules were submerged in 95% ethanol (5-10 seconds) to break the surface tension. Then, they were transferred to 3% H_2O_2 and allowed to soak for 2-3 min. After that, the nodules were rinsed in 5 to 6 changes of sterile distilled water so that there was no trace of H2O2 solution. Finally, these sterilized nodules were crushed down with a sterile glass rod with the addition of drops of sterile water. From this suspension, one loop full of suspension was inoculated on pre-sterilized Yeast Extract Mannitol Agar (YEMA) and then incubated at 28±2°C for 18 to 24 h.

Biochemical characterization

Different types of biochemical tests were performed to identify the rhizobial isolates. These tests include growth on CR-YMA agar meaning yeast extract mannitol agar with Congo red (Kneen and La Rue, 1983), starch hydrolysis test (de Oliveira et al., 2007; Bhattacharya et al., 2013), catalase and oxidase test, citrate utilization test (Simmons, 1926), MIU (Motility, Indole And Urease) test, phosphate solubilisation test (Pikovskaya, 1948), ammonia production test (Cappuccino and Sherman, 1992) and fluorescing capability test under UV light.

*nod*C and *nif*H gene amplification

Amplification of nodC and nifH genes was done in PCR tubes each containing 2.5 µL of extracted DNA (template DNA) and 10 µL Master Mix solution. The reagents used to prepare the master mix were 7.5 µL DEPC treated water, 1.0 µL 6X PCR buffer with 20 mM MgCl2 , 0.2 µL 10 mM dNTP mixture, 0.625 µL forward primer (10 mM), 0.625 µL reverse primer (10 mM) and 0.05 U Taq Polymerase. The primers used for nodC were forward 5'- CGT TTT ACG GCA AGG GCG GTA TCG GCA -3' and reverse 5'- TCC TCC AGC TCC TCC ATG GTG ATC GG -3' whereas for nifH gene the primers were forward 5'- GCC ATA GTG GCA ACC GTC GT -3' and reverse 5'- TCA CTC GCC GCT GCA AGT C -3' (Nahar et al., 2017).

EPS production and extraction

For EPS production, the isolates were inoculated into LB broth (with or without pesticides) and incubated at 30°C for seven days. EPS extraction steps begin with transferring 1 mL of previously incubated broth culture into a 1.5 mL Eppendorf tube. Then, the tube was centrifuged at 14000×g for 10 min. After centrifugation, cell-free culture filtrate (supernatant) was transferred to a fresh test tube or falcon tube of 15 mL. Three volumes of ice-cold acetone or 96% alcohol were added to one unit volume of cell-free culture filtrate for precipitating the polysaccharides and stored at 4°C for overnight. The following day, the precipitate formed at the bottom of the tube was collected and washed 3 times alternately with distilled water and then with acetone. Finally, the dissolved solution was filtered via the filter paper (0.22 µm pore sized membrane filter paper) and allowed to dry overnight at room temperature (Mukherjee et al., 2011). The filter paper was weighed the next day and the weight of the EPS was estimated.

Estimation of extracted exopolysaccharide by spectrophotometry

The dissolved polysaccharide solution was used for the estimation

Table 1. Different concentrations of pesticides.

Pesticide	Concentration (%)			
Tafgor 40EC	0.3	0.4	0.5	-
Tilt 250EC	-	-	0.5	1.0
Imitaf 20SL	-	-	0.5	1.0

of EPS by Phenol Sulphuric Acid method following Agrawal et al. (2015). To the reaction mixture in a test tube containing 1 mL of EPS solution and 1 mL of aqueous phenol, 5 mL of conc. H_2SO_4 was added. After vigorous shaking, the tubes were allowed to stand for 10 min in the dark. Then, the tubes were placed in a water bath at room temperature for 15 min. After that, absorbance was measured at 490 nm. The effects of different pesticides were evaluated on the following:

1. **Rhizobial growth:** Three different pesticides named Tafgor 40EC (manufacturer- Rallis India Limited, India; marketing company- Auto Crop Care Limited, Bangladesh), Tilt 250EC (manufacturer- Syngenta Crop Protection AG, Switzerland; marketing company- Syngenta Bangladesh Limited) and Imitaf 20SL (manufacturer- Rallis India Limited, India; marketing company- Auto Crop Care Limited, Bangladesh) were used at varying concentrations as shown in Table 1 (Ahemad and Khan, 2011).
2. **EPS production:** For evaluating the impact of pesticides at different concentrations, some representative isolates were selected based on their EPS production (Ahemad and Khan, 2011).
3. **Antibiotic sensitivity:** Bacterial suspensions (of the isolates) were prepared in Muller-Hinton broth and the turbidity was adjusted (0.5 McFarland standard) after incubation. The isolates were inoculated in Muller-Hinton Agar medium which was previously prepared with an addition of pesticide. Inhibition zone was measured in millimeter after an incubation of 24 h (Sarker et al., 2014). In this study, four antibiotics were used. These were Chloramphenicol (C), Sulfamethoxazole-trimethoprim (W), Neomycin (N) and Kanamycin (K).
4. **Phosphate solubilisation:** A modified medium, NBRIP medium (National Botanical Research Institute's Phosphate growth medium) was used to screen the solubilization efficiency (Nautiyal, 1999) with and without pesticides.

RESULTS AND DISCUSSION

Morphological characteristics

Among 42 isolates, most of them were found to be gummy and sticky, whereas only a few showed the opposite (dry colonies). Almost all of them were circular shaped except a few.

Growth on CR-YMA medium

Rhizobia growing on Yeast Extract Mannitol Agar with Congo Red produce white colonies and absorb the dye weakly. But the others (for example Agrobacterium) take up the dye strongly and form pink or orange or red-colored colonies. Here, the dye (Congo Red) actually bound with the polysaccharide portion of the rhizobial capsule (Kneen and La Rue, 1983).

Starch hydrolysis test

The starch hydrolysis test result showed that if the isolate breaks down the starch then it makes a clear zone around their colonies after adding iodine solution. This type of clear zone indicates that the isolates were capable of using starch as their sole carbon source according to Bhattacharya et al. (2013); 47.62% isolates were found to be positive for starch hydrolysis that indicates the presence of starch hydrolysing enzymes.

Oxidase, catalase and MIU test

In the oxidase test, a large portion of these isolates displayed negative results where the opposite scene was observed in catalase test results. 95.24% isolates showed positive results for catalase test by forming bubbles as reported by Shahzad et al. (2012) where 7.14% of isolates showed positive results for the oxidase test. In case of MIU test, all of the isolates were indole positive; 59.52% were motile and the rest were non-motile. The urease test result showed that about 64% isolates were able to utilize urea and break it down into ammonia, resulting in a color change while the rest of them remained yellow.

Ammonia production test

Ammonia production by these bacteria helps to influence plant growth indirectly (Geetha et al., 2014). A positive result for ammonia production exhibits yellow to brownish-yellow color (Cappuccino and Sherman, 1992). A red (sometimes brown) precipitate is an indication of the presence of ammonia because this ammonia forms an insoluble precipitate while it reacts with Nessler's reagent. The intensity of the color gradually increases with the increment of ammonia produced. 80.95% of isolates displayed blue color indicative of positive citrate utilization test as they utilize citrate as their carbon source (Gachande and Khansole, 2011).

Detection of *nod*C and *nif*H gene

Amplification of nod genes has also been reported by earlier workers and these genes have a role in nodule formation by several species of rhizobia (Haukka et al.,

1998). In this experiment, most of the isolates showed nodC 500 bp sized amplicons. Along with nodC, nifH gene (amplicon size 781 bp) was also found in most of them.

Effect of pesticides on rhizobial growth

As most of the organisms could not grow at 1% concentration of Tafgor 40EC, so the organisms were incubated to grow at 0.5% of Tafgor 40EC. The isolate R2 seemed to produce a higher amount of EPS as shown in Figures 6 and 7. To ensure how Tafgor 40EC affects the isolates, three different concentrations of Tafgor 40EC (0.3, 0.4 and 0.5%) were applied to the isolates. These three concentrations increased EPS production successively (Figure 8), but, there is another exception in R5 isolate where at 0.4% Tafgor 40EC the EPS production lowered than with 0.3% Tafgor 40EC.

Figure 9 describes that EPS production may increase or decrease when the organisms come into contact with pesticides. Here, R37 spiked up to its EPS production from its normal production when they were exposed to Tilt 250EC compared to Imitaf 20SL. But the opposite thing happened to R35 and R36 where they gradually decreased their EPS production. The comparison among sugar contents of EPS with three different pesticides shows how different pesticide contributes to different amounts of EPS production (Figure 10).

Effect of pesticides on EPS production

At first, the amount of extracted EPS of the isolates (in the absence of pesticides) was estimated and recorded. Data for some isolates have been highlighted in Table 2. Five isolates (R31, R32, R35, R36 and R37) were chosen for observing the impact of pesticides at different concentrations. But isolate R37 did not grow in the presence of Imitaf 1%. So another group of R2, R3, R4 and R5 was selected for Imitaf treatment (Figures 4 and 5).

Interestingly, the amount of EPSs secreted by these isolates increased (in certain cases decreased) as the concentrations of pesticides increased. In Figure 1, variations in EPS production by the isolates were shown where some gave a higher yield of EPS and some did a lower. The neutral carbohydrate content was evaluated by spectrophotometry method (as the EPS contains higher portions of neutral sugars in proportion to other chemicals). So, a standard curve of glucose was prepared to find out an approximate content of sugars in carbohydrates in EPS.

Figure 2 depicts that the higher concentration of Tilt 250EC (1% Tilt 250EC) increased EPS production in R37. R31 and R36 also tend to raise their EPS production (shown by the upward arrow keys) in response to the elevated concentration of Tilt 250EC when their carbohydrate contents were measured (Figure 3).

The same condition was observed in 0.5% Imitaf 20SL and 1% Imitaf 20SL (Figures 4 and 5). A similar case was reported by Ahemad and Khan (2011) where *Rhizobium* secreted EPS in higher amount when they were exposed to the pesticide-stressed environment. The exact reason behind this unusual behaviour is unknown. But, it is thought that EPSs provide protection to soil bacteria against environmental stresses; hence, it is possible that rhizobia secreted more EPSs under pesticide-stress to shield themselves against these chemicals in proportion to the pesticide-concentrations (Ahemad and Khan, 2011).

As most of the organisms could not grow at 1% concentration of Tafgor 40EC, so the organisms were incubated to grow at 0.5% of Tafgor 40EC. The isolate R2 seemed to produce a higher amount of EPS as shown in Figures 6 and 7. To ensure how Tafgor 40EC affects the isolates, three different concentrations of Tafgor 40EC (0.3, 0.4 and 0.5%) were applied to the isolates. These three concentrations increased EPS production successively (Figure 8), but, there is another exception in R5 isolate where at 0.4% Tafgor 40EC the EPS production lowered than with 0.3% Tafgor 40EC.

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Phosphate solubilisation test

As some isolates could not utilize the inorganic phosphate properly using Pikovskya's medium, a modified medium (NBRIP) was used to screen them efficiently. This time, they produced a clear halo zone around their colonies. Some of the isolates showed higher phosphate solubilisation efficiency in the presence of pesticide than the normal condition (without pesticides), while some remained the same as they were before.

Antibiotic sensitivity test

In response to pesticide, how the isolates act in antibiotic sensitivity test are summarized in the chart (Table 3). In this chart, R31 which were sensitive to Kanamycin (without pesticide) became resistant (highlighted part) in

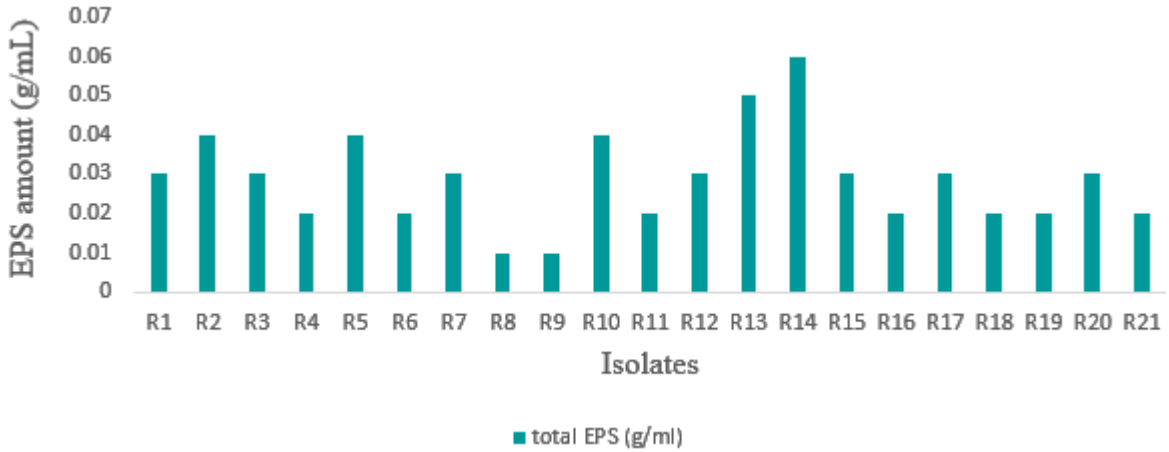

Figure 1. An illustration of different isolates producing different amounts of EPS. Data of some isolates (R1 to R21) have been presented here in the absence of pesticides.

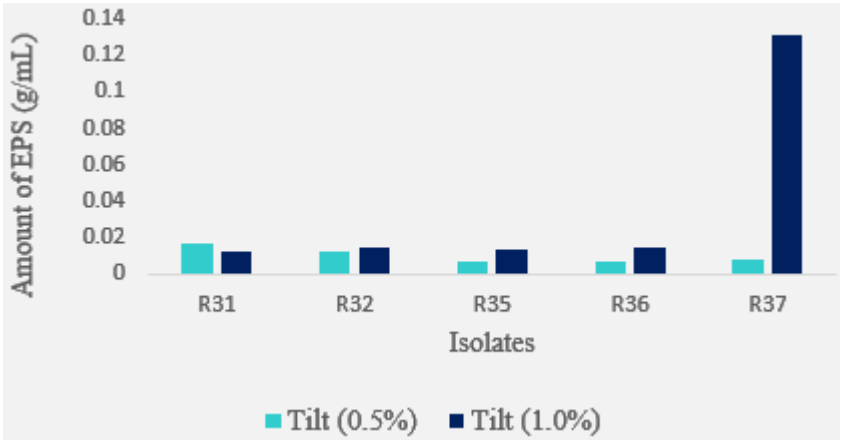

Figure 2. Effect of Tilt 250EC at two different concentrations on EPS production.

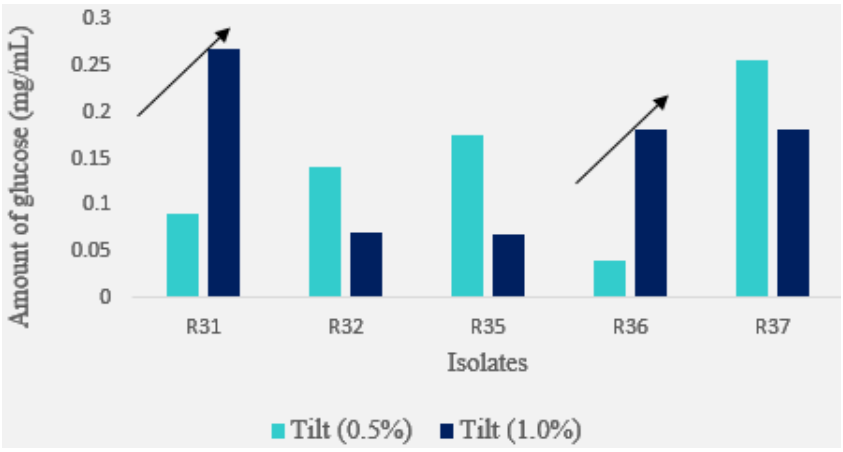

Figure 3. Effect of Tilt 250EC on EPS production (sugar content of EPS). The arrow indicates how the EPS production of R31 and R36 escalated in higher concentration of Tilt (1%). But R32, R35 and R37 showed the opposite.

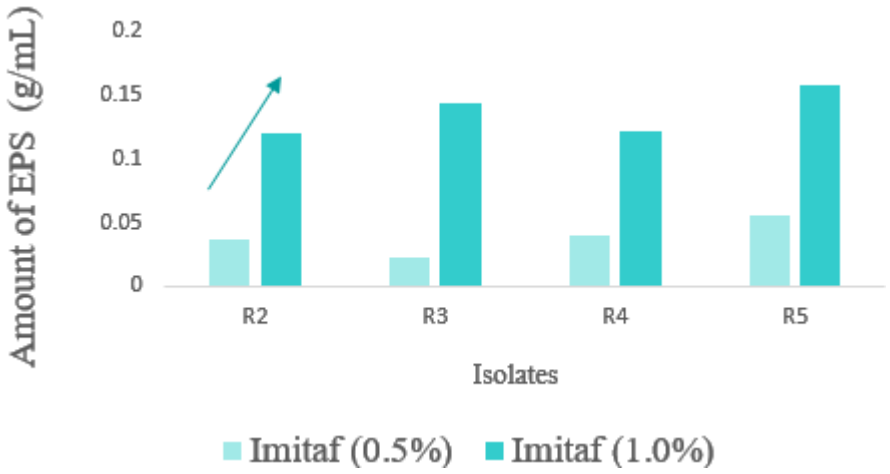

Figure 4. Effect of Imitaf 20SL on EPS production. The isolates produced a higher amount of EPS at 1% Imitaf than 0.5% Imitaf.

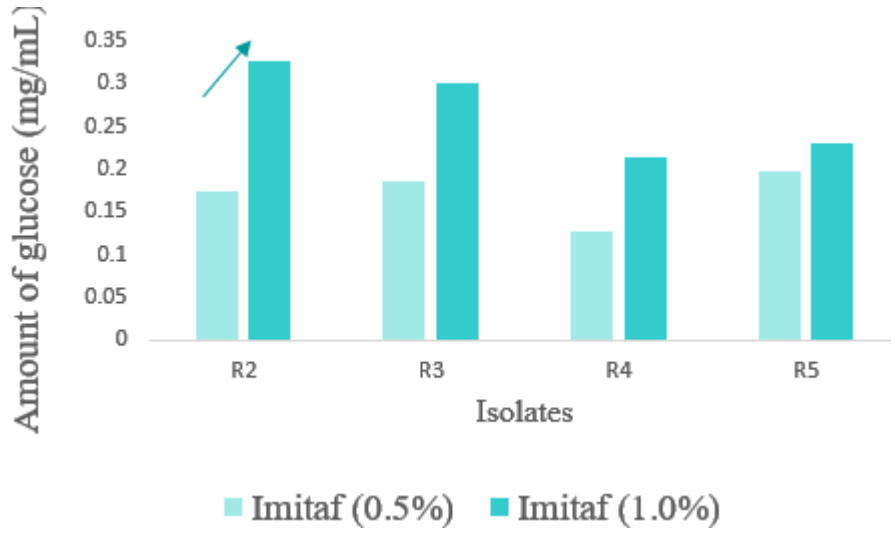

Figure 5. Effect of Imitaf 20SL on EPS (carbohydrate content). The sugar content of EPS increased in the presence of higher concentration of Imitaf (1%) than the lower one (0.5% Imitaf).

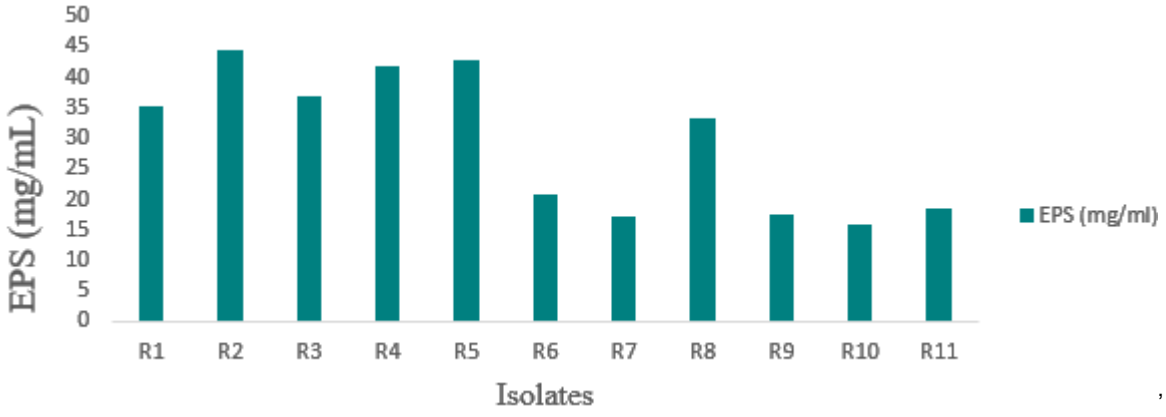

Figure 6. EPS production under the effect of Tafgor 40EC (0.5%) by different isolates.

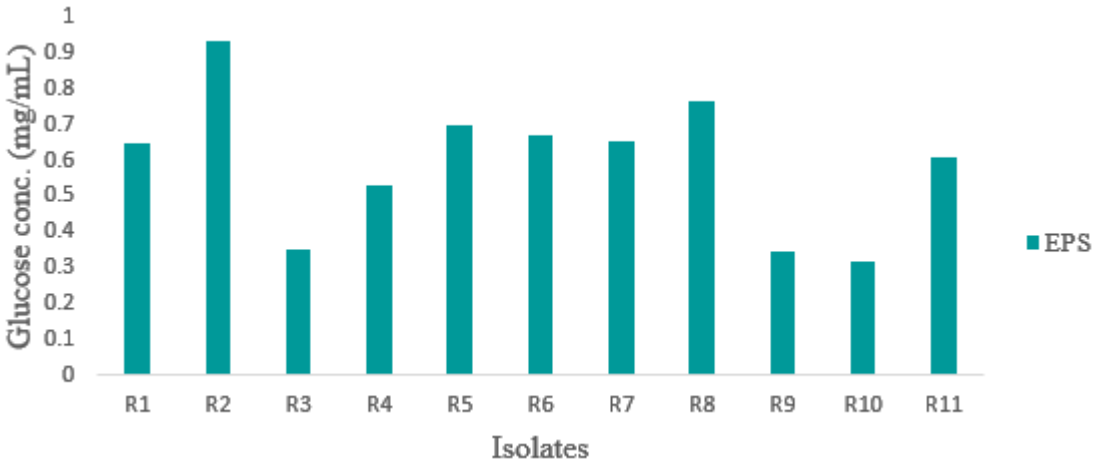

Figure 7. Sugar content in the extracted EPS at 0.5% Tafgor 40EC.

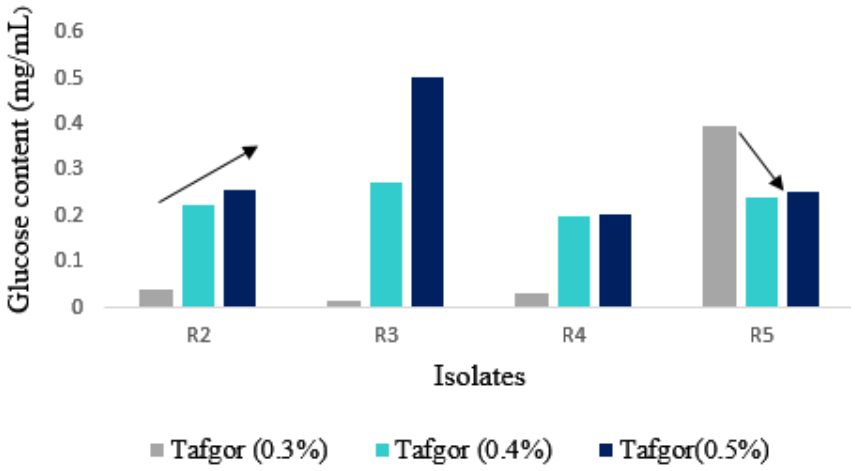

Figure 8. Variation in EPS production at different concentrations of Tafgor 40EC. At three different concentrations of Tafgor (0.3, 0.4 and 0.5%), R2 and R3 raised their EPS production. In case of R5, at 0.4% Tafgor, the EPS production lowered than 0.3% Tafgor.

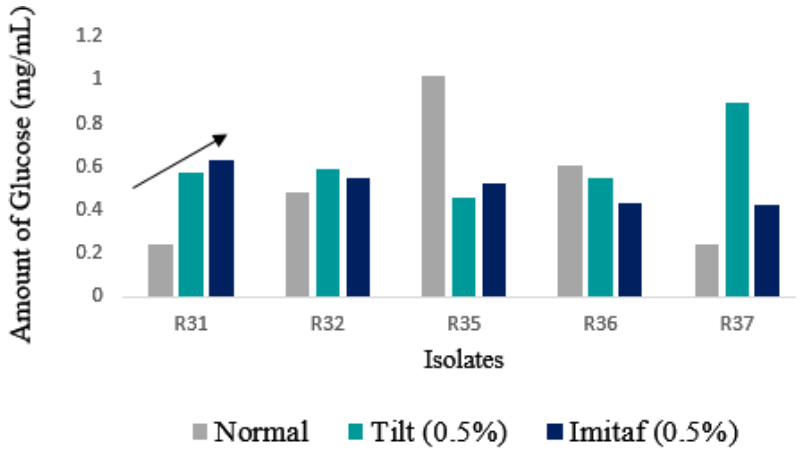

Figure 9. EPS production under normal conditions and in the presence of pesticides.

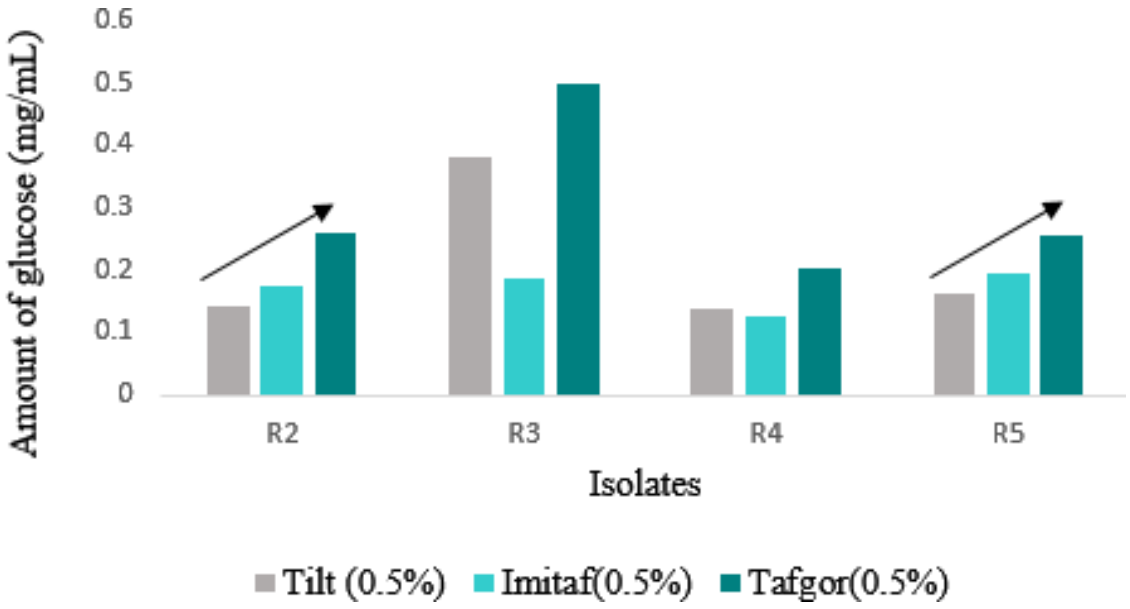

Figure 10. Comparison of EPS production under three different pesticides.

Table 2. Weight of the extracted EPS of some of the isolates (without pesticides).

Isolate	Weight of the filter paper (after extraction) (g)	Weight of the filter paper (before extraction) (g)	Weight of EPS (g/mL)
R21	0.8354	0.8143	0.0211
R22	0.8789	0.8543	0.0246
R23	0.8228	0.8014	0.0214
R24	0.8639	0.8413	0.0226
R25	0.8815	0.8582	0.0233
R26	0.8581	0.8369	0.0212

the presence of 1% Tilt 250EC. Here, the isolate R35 was sensitive to Kanamycin (in the presence of 0.5% Imitaf) but it became resistant to Kanamycin in the presence of 1% Tilt. Additionally, there was an upgrade from ‘sensitive’ to ‘resistant’ to Kanamycin exerted by R37 when it was exposed to Tilt 0.5% to Tilt 1%, respectively. So, based on the results from sensitivity to antibiotics, it could be said that the isolates developed resistance because of these stressed conditions and thus switched from sensitive to resistant, gradually.

Conclusion

EPS production varies greatly upon the type and concentration of the pesticide. Continuous delivery of these pesticides in the soil might not give a profitable output in case of growth of rhizobia and other distinguishing characteristics like EPS production, antibiotic susceptibility and phosphate solubilisation. The isolates may be further tested to observe antimicrobial activity and the impact of pesticides on other characteristics like siderophore production. So considering all the impacts of pesticides, awareness about the usage of pesticides must be raised to preserve both the environment and living organisms.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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Table 3. Antibiotic sensitivity under different concentrations of pesticides.

Antibiotics	Chloramphenicol (C)	Sulfamethoxazole trimethoprim (W)	Neomycin (N)	Kanamycin (K)
R31	S	S	R	S
Imitaf (0.5%)	S	S	I	S
Imitaf (1.0%)	I	I	I	I
Tilt (0.5%)	S	S	I	I
Tilt (1.0%)	I	I	R	R
R32	S	S	R	I
Imitaf (0.5%)	S	S	I	S
Imitaf (1.0%)	S	I	I	R
Tilt (0.5%)	S	I	I	I
Tilt (1.0%)	S	S	R	R
R35	S	S	R	I
Imitaf (0.5%)	S	S	R	S
Imitaf (1.0%)	S	I	I	I
Tilt (0.5%)	I	I	R	I
Tilt (1.0%)	I	I	R	R
R36	S	S	I	R
Imitaf (0.5%)	S	S	R	S
Imitaf (1.0%)	I	I	I	I
Tilt (0.5%)	S	S	R	I
Tilt (1.0%)	I	I	R	R
R37	S	S	I	I
Imitaf (0.5%)	S	S	I	I
Imitaf (1.0%)	-	-	-	-
Tilt (0.5%)	S	S	I	S
Tilt (1.0%)	I	I	R	R

S, Sensitive; R, Resistant ; I, Intermediate.

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