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ASIAN JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN: 0976-3376

Asian Journal of Science and Technology
Vol. 11, Issue, 08, pp.11127-11131, August, 2020

RESEARCH ARTICLE

INOCULATION OF EXOPOLYSACCHARIDE–PRODUCING AZOTOBACTER FOR INCREASING BACTERIAL POPULATION IN RHIZOSPHERE AND YIELD OF SOYBEAN IN POT EXPERIMENT

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ARTICLE INFO

Article History:

Received 17th May, 2020
Received in revised form
09th June, 2020
Accepted 24th July, 2020
Published online 30th August, 2020

Key words:

Azotobacter, Exopolysaccharides, Nodulation, Nutrient Uptake, Soybean

ABSTRACT

Azotobacter is well known plant growth promoting rhizobateria that fix diniitrogen (N_2), and produce phytohormones and exopolysaccharides (EPS). The objective of this pot experiment was to evaluate the effect of Azotobacter liquid inoculant as well as its crude EPS on N_2 fixing bacteria population, essential macronutrientsst uptake and yield of soybean cv. Anjasmoro grown in potted soil. The experiment was arranged in randomized block design with five replications. The combination treatments consisted of two doses of Azotobacter two doses of crude EPS and two doses of liquid inoculant of Azotobacter. All plants were also inoculated with *Bradyrhizobium* sp. isolated from the rhizosphere of said soybean. Results showed that liquid inoculant and crude EPS of Azotobacter enhanced total bacteria and Azotobacter population in the rhizosphere as well as root nodule number. The experiment verified that nitrogen and phosphate uptake by soybean shoots increased by both treatments, but there was no change in potassium uptake. Grain yield of plants inoculated with Azotobacter and crude EPS were higher than the control.

Citation: ***Reginawanti Hindersah and Arief Harsono*. 2020.** "Inoculation of exopolysaccharide–producing Azotobacter for increasing bacterial population in rhizosphere and yield of soybean in pot experiment", *Asian Journal of Science and Technology*, 11, (08), 11127-11131.

INTRODUCTION

Azotobacter is plant growth promoting rhizobacteria (PGPR) through the mechanisms of N_2 fixation (Kizilkaya, 2009; Santi *et al*., 2013), phytohormones production (Hindersah *et al*., 2009; Rubio *et al*., 2013), and EPS production (Vermani *et al*. 1997; Hindersah *et al*., 2011). The ability of Azotobacter to produce EPS has been reported elsewhere. Non-symbiotic N_2-fixer Azotobater synthesize EPS to withstand drought, abiotic stress and protect nitrogenase from oxygen (Sabra *et al*., 2000; Abd El-Ghany and Attia, 2020). Advantage of using EPS of Azotobacter on nutrient uptake through several mechanisms is also documented. Exopolysaccharides of rhizobia play a significant role in the soil structure stability through aggregation and soil pore formation (Alami et a., 2000) and increase nutrient uptake especially Nitrogen (N). The increase of weight of root-adhering soil due to EPS were reported to increase rhizosphere soil in which the uptake of nutrient by plant roots is become intensive due to among other microbial function (Amellal *et al*., 1998; Costa *et al*., 2018). Plant growth promoting rhizobacteria include Azotobacter was associated with their proliferation in the rhizosphere to produce EPS. In turn the EPS might stimulate the growth of plant-beneficial microbes in the rhizosphere (Gauri *et al*., 2012) since EPS contained carbohydrate as well as organic acid as microbail nutrients (Vargas Garcia *et al*., 2003; Hindersah, 2015). Exopolysaccharides of Azotobacter protect rhizosphere microbes from environmental stress by providing the energy and carbon source for their heterotrophic anabolism. The use of EPS-producing Azotobacter might be a significant and novel way to increase soybean production in Indonesia since the soybean productivity is still low. Moreover, soybean is the main ingredient for making tofu and fermented-food *tempe*, both are the main source of protein in daily diet of Indonesian communities. Co-inoculation of EPS-producing Azotobater and Bradyrhizobium in legumes especially soybean is a promising method to enhance soybean yield. A field trial revealed a significant increase in nodulation and grain yield of soybean up to 42% following dual inoculation of Rhizobium and Azotobacter (Nandi *et al*., 2013). Chemical analysis of lentil above ground parts showed significantly high content of N (4.4%) due to co-inoculation compared to rhizobia alone (4.21%) under chemical fertilization (Akhtar *et al*., 2012).

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Microbes synthesize EPS from glucose catalyzed by series of *lgn* gene and excrete them to outer environmental either as soluble or insoluble polymers (Remminghorst and Rehm, 2006; Sivakumar *et al*. 2012). In liquid culture, extracellular slime of Azotobacter can be recovered by centrifugation by acetone (Hindersah *et al*., 2011) so that EPS might be applied to soil in plant production. Our preliminary research showed that utilization of crude EPS on soybean increased Bradyrhizobium population in the rhizosphere and induced the formation of effective nodules at 21 days after sowing. In order to verify the effect of Azotobacter's crude EPS on soybean, pot experiment was carried out. The objective of the pot experiment was to verify the influence crude EPS and liquid inoculant of rhizobacteria Azotobacter on N_2 fixing bacteria and total bacteri population nodule number, N, P and K uptake and yield of soybean cv. Anjasmoro grown in potted soil.

MATERIALS AND METHODS

Pot experiment was performed in experimental field belong to Faculty of Agriculture Universitas Padjadjaran, Jatinangor Campus at 750 m above sea level. Jatinangor is located in tropical regions with average rainfall of 2,188 mm per year. The potted soil was Inceptisol taken up from the top soil of agricultural area in the campus. The soil was silty clay with the acidity of 5.83. The soil chemical properties were 1.65% total organic carbon, 0.16% total nitrogen, C/N 10.18, 33.51 mg/100 g total P, 24.17 mg/kg available P_2O_5, 15.31 mg/100 g K_2O; soil had medium cation exchange capacity and high base saturation. Based on chemical properties, the soil was not fertile but the texture might support root growth. Soil carbon was low so that cow manure should be added. The manure contained 33.5% carbon organic, 1.8% total nitrogen, C/N 18, water content 22.34%; the *Escherichia coli* and *Salmonella* sp. count that were lower than 10^3 MPN/g agrees with Indonesian regulation for agricultural organic matter. The manure was produced by Waste Treatment Unit in Faculty of Animal Husbandry Universitas Padjadjaran.

Liquid Inoculant and EPS of Azotobacter Preparation

The N_2-fixing bacteria *A. chroococcum* is a collection of Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran and isolated from rhizosphere of maize grown in Inceptisol. Bacterial pure culture was maintained in N-free Ashby's mannitol slant. Soybean (*Glycine max* (L) Merill) cv Anjasmoro were provided by The Indonesia Legume and Tuber Research Institute. Liquid inoculant of *A. chroococcum* was scaled up by using 1% molasses enriched with 0.1% ammonium chloride in 2 L glass fermenter. A total of 1L molasses-based medium was autoclaved for 20 minutes at 121°C prior to inoculate by 5% Azotobacter liquid pure culture at the density of 10^8 CFU/mL. Inoculated liquid medium was poured into aseptic fermenter equipped with 115 rpm rotary shaker at room temperature for 3 days. At the end of incubation, 500 mL of Azotobacter liquid culture was centrifuged 10,000 rpm at 4 °C for 20 min. The supematant was collected as crude EPS and used for the experiment.

Experimental Set Up: Pot experiment was carried out in completely block design which test five treatments (Table 1) that replicates five times. Two units of experiments was performed.

Table 1. Azotobacter Crude Exopolysachharides and liquidinoculant treatments for pot experiment

Treatments	Concentration
Control (without Azotobacter)	
10 mL of crude EPS	14 g/L
20 mL of crude EPS	14 g/L
10 mL of liquid inoculant	10^8 CFU/mL
20 mL of liquid inoculant	10^8 CFU/mL

The top soil of Inceptisol was air dried and sieved by using 5 mm opening sieve. A total of 8 kg soil was putted into black plastic container where the soil then mixed with cow manure as much as 80 g/pot equivalent of 20 t/ha. Potted soils were irrigated with ground water for reaching field capacity. The surface of soybean seeds was sterilized by immersing in calcium hypochlorite solution for 30 seconds before washing triplicate with sterilized aquadest. After washing, 100 seeds were mixed with 200 mL *Bradyrhizobium* sp. liquid inoculant with the density of 10^7 CFU/mL for one hour. Crude EPS and liquid inoculant were applied by pouring said volume on the 5 cm depth hole soon before sowing two soybean seeds into the hole and covered with soil. Urea, SP-36 and Potassium Chloride fertilizers at the rate of 50% of recommended doses were applied at 5 cm away from plant hole soon after sowing. All pots were placed outdoor without shade to avoid etiolation of soybean shoot once they grow.

At 42 days after sowing, shoots from the first unit was separated from the roots. Rhizosphere soil was collected from each roots, soil in each pot was mixed evenly. A total of 1 g of rhizosphere soil and 10 g of bulk soil was diluted using 0.85% sodium chloride before Azotobacter total bacteria population count. Bacterial enumeration was carried out by serial dilution plate method in N-free Ashby agar for Azotobacter and Nutrient Agar for total bacteria. The plate culture was incubated for 48 h at 30 °C. Effective nodules that have diameter greater than 2 mm and pink in color were counted from roots. Pink nodule indicates the presence of leghemoglobin that is important for protecting nitrogenase from the oxygen. Plant shoots were heated at 70 °C for two days to obtain their dry weight and delivered to laboratory for N, P and K content analysis prior to N, P, and K uptake calculation by multiplying the biomass with the nutrient content. At harvest time, 92 days after sowing, plant yield was measured in total grain weight. The quality of grain was determined by dry weight of 25 seeds. The data were subjected to analysis of variance ($p<0.05$). If the sum square of the treatment on certain traits were significant then Duncan's multiple range test ($p<0.05$) was performed.

RESULTS AND DISCUSSION

Azotobacter and Total Bacteria Population in Soybean Rhizosphere: Duncan's test showed that Azotobacter treatments had a significant effect on total bacterial and Azotobacter count in the soybean rhizosphere but did not in bulk soil (Table 2). Before experiment, the number of Azotobacter and total bacteria in untreated soil was 2.75×10^5 CFU/g and 1.72×10^4 CFU/g respectively. At the end of vegetative phase, 42 days after sowing, rhizosphere soil contained at least 10^{10} CFU/g total bacterial and 10^8 CFU/g Azotobacter irrespective of the treatments. Rhizosphere of soybean that received crude EPS clearly contained higher total bacteria and Azotobacter population as well as bacterial ratio

Table 2. Effect of crude EPS and liquid inoculant of Azotobacter on total bacteria and Azotobacter population in rhizosphere and bulk soil of soybean at 42 days after sowing

Azotobacter treatments	Rhizosphere		Bulk soil		R/S TB*	R/S Azotobacter
	TB* (10^{10} CFU/g)	Azotobacter (10^8 CFU/g)	TB* (10^{10} FU/g)	Azotobacter (10^8 CFU/g)		
Control	7.28 a	2.54 a	2.63	0.94	2.7a	2.7a
10 mL crude EPS	15.59 c	4.89 b	2.91	1.11	4.3b	4.4b
20 mL crude EPS	10.86 bc	4.71 b	2.72	1.12	3.9b	4.2b
10 mL liquid inoculant	9.72 b	6.76 c	2.65	1.16	3.7b	5.8ab
20 mL liquid inoculant	10.35 b	6.59 c	2.89	1.05	3.6b	6.3ab

Number followed by the same letter in column was not significantly different based on Duncan's Multiple Range Test ($p<0.05$). *TB: total bacteria

Table 3. Increased in nitrogen, phosphor and potassium uptake of aerial part of soybean grown with either crude EPS or liquid inoculant at 42 days after sowing

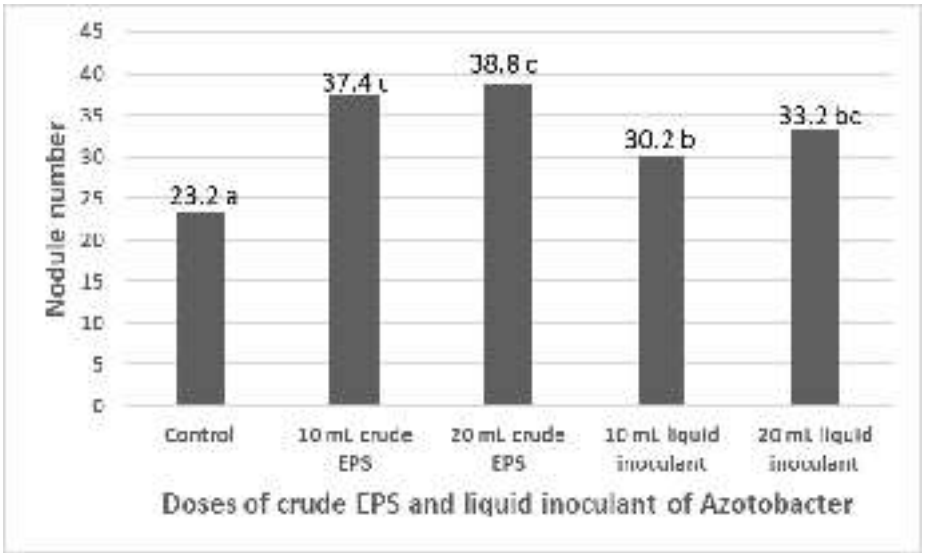
Azotobacter treatments	Nutrient uptake (mg/plant)		
	Nitrogen	Phosphor	Potassium
Control	3.9 a	0.10 a	0.98 a
10 mL crude EPS	10.9 c	0.37 c	2.01 b
20 mL crude EPS	9.6 bc	0.22 b	1.27 a
10 mL liquid inoculant	6.8 ab	0.25 b	1.23 a
20 mL liquid inoculant	6.6 ab	0.21 b	1.12 a

Number followed by the same letter in column was not significantly different based on Duncan's Multiple Range Test ($p<0.05$)

Table 4. Effect of crude EPS and liquid inoculant of Azotobacter on grain weight per plant and weight of 25 grains of soybean at harvest time

Azotobacter Treatments	Grain weight per plant (g)	Weight of 25 grains (g)
Control	6.43 a	4.0 a
10 mL crude EPS	8.61 b	4.3 a
20 mL crude EPS	8.26 b	4.2 a
10 mL liquid inoculant	8.90 b	4.3 a
20 mL liquid inoculant	9.26 b	4.5 a

Number followed by the same letter in column was not significantly different based on Duncan's Multiple Range Test ($p<0.05$)



Number followed by the same letter in column was not significantly different based on Duncan's Multiple Range Test ($p<0.05$)

Fig 1. Effect of crude EPS and liquid inoculant of Azotobacter on nodule number of soybean at 42 days after sowing

in rhizosphere to bulksoil (R/S). This indicated that plant received Azotobacter resulted in healthy rhizosphere. Plant roots released variety of chemical compounds to attract and select microbes in rhizosphere (Huang *et al*., 2014). Root exudates that contained mainly organic substances initiate and modulate dialogue between roots and soil microbes (Badri and Vinanco, 2009). Healthy rhizosphere was colonized by a diverse species of microbes, biological interaction between them as well as between microbes and roots could form dynamic microbial community structure which in turn provide benefit for plant (Widyati, 2016). Our previous pot experiment showed that crude EPS induce Bradyrhizobium population in the rhizosphere of soybean (Sara, 2014). Crude EPS and Liquid inoculants of Azotobacter did not induce total bacterial count compared with the control but Azotobacter population was significantly higher in soybean that received crude EPS and liquid inoculant (Table 2). The results verified that exogenous Azotobacter enable to survive in soybean rhizosphere and performed synergistic effect with the indigenous one. Increasing Azotobacter population in the

rhizosphere of soybean was due to presence of root exudates that assure proliferation of either exogenous or indigenous Azotobacter. In this experiment the total bacteria might include beneficial and harmful bacterial. However, during the experiment, there was no soil borne diseases incidence suggested that harmful bacteria did not dominate.

Number of Effective Nodule: Based on Duncan's Test, the effect of Azotobacter treatments on nodule number was significant. Number of nodule in soybean was clearly greater in plants treated with EPS and liquid inoculant of Azotobacter compared with the control (Table 3). Increased of effective nodule in treated plant agreed with increase of Azotobacter population in the rhizosphere (Table 2). Effective nodule observation was carried out at the end of the vegetative phase when roots uptake the maximal nutrients so that nodulation also achieves optimal phase. Interaction between rhizobia and legume root involved the flow of carbon from root cells to the rhizobia in nodule. Increased in nodule number following either crude EPS application or Azotobacter inoculation was due to rhizobia immobilization in the rhizosphere by EPS which in turn induce nodule formation (Nandi *et al*., 2013). Higher nodule number in plant treated with either EPS application or Azotobacter inoculation agreed with the increase of Azotobacter population (Table 2) resulted in more EPS in rhizosphere. Although liquid inoculant centrifugation to obtain crude EPS was taken place, there was 10^2 CFU/g Azotobacter in the crude EPS that enable to proliferate in the rhizosphere and produce EPS.

Nutrient Uptake and Yield: Crude EPS and Azotobacter inoculant increased N and P uptake significantly compared to the control (Table 3). Only plant treated with 10 mL of crude EPS has K uptake higher than control and other treatments. Increased of macronutrients uptake mainly N and P by aerial part of soybean might be caused by improvement of soil physics in the rhizosphere. Exopolysaccharides–producing *Pantoea agglomerans* NAS206 regulated the water content (excess or deficit) of the wheat rhizosphere by improving aggregation (Amellal *et al*., 1998). Beneficial effect of EPS of *Rhizobium* to increase nutrient uptake by roots of sunflower are also documented (Alami *et al*., 2000). In this research, the contribution of Azotobacter through nitrogen fixation to N uptake is demonstrated. Soybean grown with crude EPS or Azotobacter liquid inoculant had higher grain weight per plant (Table 4).

The results showed that the grain weight of plant with any dose of EPS did not significantly different with plant received any dose of Azotobacter liquid inoculant. There was no significant difference between the weight of 25 grains indicated that perhaps the quality of seed was similar. Grain weight per plant that received 20 mL of liquid inoculant (9.26 g) was highest among the treatments which agreed with the higher ration of Azotobacter in rhizosphere to bulk soil (Table 2) and nodule number (Fig 1). The increase of grain weight per plant with said treatment is 44% compared to the control, although statistically did not significantly different with the other treatments The results of this experiment was in accordance with the increased of grain yield of soybean up to 42% in a field trial after dual inoculation with rhizobia and Azotobacter (Nandi *et al*., 2013). Other experiment showed that mixed inoculation Bradyrhizobium, Azotobacter and Bacillus increased the number and weight of nodules and the biomass u to 22 %-105% (Melnykova *et al*., 2020).

Conclusion

The abundance of total bacteria and Azotobacter in rhizosphere of soybean treated with either crude EPS or liquid inoculant of Azotobacter was higher than the control. This verified that the Azotobacter treatments resulted in healthier rhizosphere which is also proved by higher nodule number. Increased in N, P and K uptake was evidence in soybean shoots received 10 mL crude EPS of Azotobacter. Grain weight per plants inoculated with Azotobacter and crude EPS were higher than the control although the weight of 25 grains was not affected by both treatments.

Acknowledgement

Authors thanks Rifqi Rachmawan and Suryo Nurasyid Santoso who assisted the experiment. The research fund comes from Indonesian Higher Education General of Ministry of Education year 2016.

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