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Research Article
Plant Developmental Stages Effect on Antifungal Activities of Fluorescent *Pseudomonas* under Controlled Environment

[1]Eshetu Yilma and [2]Tesfaye Alemu

[1]Department of Plant Sciences, Salale University, Fitche, Ethiopia
[2]Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, Ethiopia

Abstract

Background and Objective: The status of the rhizosphere scheme changes on the basis of variables such as age of the plant, root area, temperature and others. Hence, this study is initiated to evaluate the effects of plant developmental age on antifungal activities of rhizospheric fluorescent *Pseudomonas*. **Materials and Methods:** The soil samples were collected from 0-20, 0-40 and 0-60 cm depth from cambisol soil, mixed thoroughly and filled in a pot. Then crops were planted in pot and sampling from the planted root area was taken 9 times at different developmental stages (every 15 days interval) and density and antifungal activity of fluorescent *Pseudomonas* were examined. **Results:** Out of these plant developmental stages, the highest density (using standard plate count method) and strongest antifungal activity of fluorescent *Pseudomonas* (by measuring growth inhibition zone on nutrient agar plate for the 72 h incubation at 37°C) were observed for both samples from *Vicia faba* L. and *Zea mays* L. When the age of the crops was 91 days after being planted in a pot, both crops were not flowered entirely but almost were ready. The density and antifungal activity were best recorded for *Vicia faba* L. for the soil sample 0-60 cm depth. In case of control pots (without planting), less (no) antifungal activity was seen while the density of the bacterium was declined until 76 days and after 76 days density remained same. **Conclusion:** This finding highlighted that considering the plant developmental stage during sampling is relevant in the process of isolating and identifying potential bacterial bio-control agents.

Key words: Bio-control agent, isolation, identification, crops, fungi, developmental stage, fluorescent *Pseudomonas*

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Corresponding Author: Tesfaye Alemu, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, Ethiopia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The rhizosphere is the soil influenced due to biological and biochemical activities by the living root[1]. The German agronomist Hiltner first defined the rhizosphere in 1904 as the 'effect' of the roots of legumes on the surrounding soil, in terms of higher microbial activity because of the organic matter released by the roots. The rhizosphere is the biggest ecosystem on earth and the energy flux in this rhizosphere is massive. Some authors estimate that plants release between 20 and 50% of their photosynthetic product through their roots[2,3]. The rhizosphere is an ultimate environment for many organisms and communities. Especially the rhizosphere of wild plant species appears to be the preeminent source from which to screen plant growth-promoting rhizobacteria (PGPR) and this is because it supplies water, oxygen, organic substrates and physical protection[4]. The plant exudates consist of amino acids, sugars, organic acids, proteins, polysaccharides, growth promoting, growth inhibiting substances, fatty acids, nucleotides, organic acids, phenolics and vitamins[5]. The organic carbon source and the binding surface provide a vibrant energy for the colonization of microbes in the rhizosphere and create the rhizospheric effect[6-9]. It is estimated that, about 2-5% of rhizosphere bacteria are plant growth-promoting[10]. A particular bacterium is categorized as PGPR when it is capable of producing a beneficial effect on the plant after inoculation, hence showing significant competitive abilities over the existing rhizosphere communities. One of those rhizosphere microbial communities is bacteria occupying the soil around the root and being useful to plants which are referred to as plant growth promoting rhizobacteria (PGPR)[11].

From PGPR, so far *Pseudomonas* is the chosen one because of its desirable characteristics, particularly its antifungal activities. *Pseudomonas* is the genus which belongs to the γ subclass of the *Proteobacteria* and in most cases it includes fluorescent *Pseudomonas* as well as a few non-fluorescent species[12]. Fluorescent *Pseudomonas* represents (i) phytopathogenic cytochrome c oxidase-positive species, such as *P. cichorii*, *P. marginalis* and *P. tolaasii* (ii) Non-phytopathogenic, non-necrogenic strains, such as *P. fluorescens*, *P. putida*, *P. chlororaphis*, *P. aureofaciens* and *P. aeruginosa* depending on species (iii) Phytopathogenic necrogenic fluorescent *Pseudomonas* spp. without cytochrome c oxidase: *P. syringae* and *P. viridiflava*. Non-fluorescent *Pseudomonas* group constitute of *P. stutzeri*, *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes*[13-15]. *Pseudomonas aeruginosa* is known to be an opportunistic human pathogen that causes chronic infections with cystic fibrosis.

Some of the PGPR have been commercialized as inoculants for agriculture but it must be borne in mind that the inoculation of these bacteria in soil may affect the composition and structure of microbial communities and these changes must be studied since they have, at times, been related to the inefficiency of bio-fertilizers when applied to plant roots[16]. On the other hand, several studies evaluated the effectiveness of PGPR in a different situation, confirming that PGPR is efficient mostly under controlled environment[17]. The interaction with the biotic factor is vital because PGPR must occupy a new niche, sticking to the plant roots and the inoculum must battle for available nutrients released particularly by the root exudates, upholding the lowest number of inhabitants capable of exerting its biological influence.

The status of the rhizosphere scheme changes on the basis of variables such as the age of the plant, root area, light availability, humidity, temperature and plant nutrition[18,19]. Under the stressful condition, the plant exerts a stronger control on the release of exudates[20-22] to assume that, changes occur in the plant may influence the root exudation patterns reasonably. There have been many studies that associate with the quality and quantity of exudates with changes in the structure of rhizosphere microbial communities[23]. It is clearly indicated that there are no research findings that directly link the plant age with the antifungal activities of rhizospheric bacteria. Therefore, the current study is initiated to evaluate the effects of the plant developmental age on one of the rhizospheric microbes (fluorescent *Pseudomonas*) in terms of its antifungal properties.

MATERIALS AND METHODS

Research procedure: This pot experiment was conducted from October 01, 2015 to June 30, 2016 in a glass house at Raaree Research site in Haramaya University located at 520 km away from Addis Ababa in the eastern part of Ethiopia. The soil was collected from 50 m^2 where the latitude is 9°24′ N, longitude 42°03′ E and altitude 2006 m.a.s.l. The soil was taken from 0-20, 0-40 and 0-60 cm depth (from the cambisol soil) and for each depth, sufficient amount of soil sample to the pot size was taken from that particular site and mixed thoroughly. The treatments were 14, (3 soil depth, 9 developmental stages of the crops and 2 crop types) indicated as (0-20, 0-40, 0-60 cm), (0, 16, 31, 46, 61, 76, 91, 106, 121 days after planting), legume (*Vicia faba* L.) and non-legume (*Zea mays* L.), respectively. Completely randomized design (CRD), with 3 replications/treatment was used. Before the crops were planted in a pot, 10 g of soil was taken from all the samples, diluted serially and growth of

microorganisms was assessed on King's B medium (KBM) and Nutrient agar medium for bacteria and Czapek Dox's Agar (CDA) for fungi to roughly see which microorganism growth in this soil environmental favors. So, for *Vicia faba* L. 12 pots (1 control, 3 replications for each depth) and *Zea mays* L. 12 pots (1 control, 3 replications for each depth), total 24 pots were utilized in this experiment. In the control, no crop was planted in the pot, instead, the dried stick was put at the center. The crops were irrigated uniformly and their developmental stages were captured at every 15 days interval. Simultaneously, the laboratory analysis on the density, morphological characteristics and antifungal activity of fluorescent *Pseudomonas* was performed for the sample taken from the pot of both crops and the control at every 15 days interval. The data were analyzed using Microsoft Excel 2010.

Isolation of the pathogenic fungi: *Fusarium* spp. was isolated from the soil of Raaree potato fields (Research site in Haramaya University). One gram of soil was dissolved in 9 mL of sterilized distilled water and 1ml is serially transferred from 10^{-1} to 10^{-5}dilution factor. Czapek Dox's Agar medium (CDA) is used at a dilution factor of 10^{-4} for pour plating method. Chloramphenicol is added to CDA to restrict both Gram +ve and Gram -ve bacteria and finally kept in an incubator at 27°C. All the procedures were carried out under the laminar hood. After 6 days of incubation, small colonies of fungus appeared, which were picked with a sterilized toothpick and transferred to Potato Dextrose Agar Medium plates and then kept on sterile glycerol for maintenance. Few types of *Fusarium* spp. were identified based on colony morphology, morphological characteristics of macro and microconidia and conidial measurement. Among these *Fusarium* isolates, *Fusarium oxysporum* species were identified using a manual of Booth[24]. The conidial measurement was carried out using a microscopic image at 400X magnification.

Isolation of fluorescent *Pseudomonas*: The rhizospheric soil samples were collected in an envelope at 15 days interval as stated in the methodology from the pot where *Vicia faba* L. and *Zea mays* L. were planted and brought to plant protection laboratory, School of Plant Sciences, Haramaya University. Isolation of fluorescent *Pseudomonas* was carried out on King's B medium (KBM)[25]. One gram of soil was dissolved in 9 mL of sterilized distilled water. One milliliter is serially transferred from 10^{-1} to 10^{-6} dilution factor. Samples were serially diluted and taken from 10^{-6} dilution factor and 0.1 mL of sample was spread on King's B medium plates. After incubation at 28°C for 48 h, the plates were exposed to UV light at 365 nm for few seconds and the colonies exhibiting the fluorescence were picked up and streaked on to the slants for maintenance, purified on King's B medium plates for latter antimicrobial activity test.

Antifungal activity of fluorescent *Pseudomonas*: *Fusarium* spp. was invigorated in nutrient broth. Then, 200 µL of *Fusarium* spp. culture was spread on nutrient agar plates. Using inoculating loop, a loop full from pure bacterial culture (i.e., fluorescent *Pseudomonas*) was placed on the surface of the fungus inoculated nutrient agar plate and incubated at 37°C for 72 h. Three of the bacterial isolates designated as FP-1, FP-2 and FP-3, were selected based on their fungus inhibiting potential and then processed for optimization of their growth conditions, characterization and identification. FP is just to say fluorescent *Pseudomonas*. In general, the characterization of the colony morphology was performed as per the protocol of phenotypic characterization[26].

Statistical analysis: The experiments were conducted in completely randomized design (CRD) with 3 replicates/ treatment. The data were analyzed using Excel 2010 and significance between control and treatment was compared at 0.05 probability levels.

RESULTS

Morphological characterization (color, shape and structure) of the colony of fluorescent *Pseudomonas* for the sample from *Vicia faba* L.: The observation was made by the naked eye and dissecting microscope for all the samples collected from the crop *Vicia faba* L. In this process, the emphasis has been given to the medium type used and growth condition since those are highly influencing the morphology of the colony. Then the diameter of the colony was measured with a ruler (mm) and found to be small, medium or large (not reported) as shown in Table 1.

Odor, density and antifungal activity of fluorescent *Pseudomonas* for the sample from *Vicia faba* L. Antifungal activity of the bacterial isolates against *Fusarium* spp. was recorded. Growth inhibition zone (GIZ) of fluorescent *Pseudomonas* (for isolate FP-2 after 24 h) was observed to be 3 mm which increased to 5.17 and 8 mm after 48 and 72 h of incubations, respectively. In case of other isolates, no growth inhibition zone was observed after 24 h but the zones were noticed as 1.5, 2.5, 1.8 and 2 mm after 48 and 72h of incubation for FP-1 and FP-3, respectively. FP = fluorescent *Pseudomonas* as shown in Table 2.

Table 1: Morphological characterization of the colony (color, shape and structure) of fluorescent *Pseudomonas* sampled at different developmental stages of the crop *Vicia faba* L.

Faba bean (*Vicia faba* L.) age after sowing (days)	Colony color of fluorescent *Pseudomonas*	Colony shape of fluorescent *Pseudomonas*	Colony structure of fluorescent *Pseudomonas*
0	Yellow	Circular	Opaque
16	White mucus	Irregular	Translucent
31	Creamy	Rhizoid	Transparent
46	Creamy	Circular	Opaque
61	Yellow-green	Irregular	Translucent
76	White mucus	Rhizoid	Transparent
91	White mucus	Circular	Opaque
106	White mucus	Irregular	Translucent
121	White mucus	Irregular	Translucent

Result indicates that at the crop (*Vicia faba* L.) age of 31 days after planting, the color, shape and structure of the bacterial colony (fluorescent *Pseudomonas*) was found to be creamy, rhizoid and transparent, when the crop age is 91 days after planting, the color, shape and structure of the bacterial colony (fluorescent *Pseudomonas*) was found to be white mucus, circular and opaque for the samples taken from the same crop (*Vicia faba* L.)

Table 2: Characterization of the odor, density and antifungal activity of fluorescent *Pseudomonas* sampled at different developmental stages of the crop *Vicia faba*

Faba bean (*Vicia faba* L.) age after sowing (days)	Colony odor of fluorescent *Pseudomonas*	Density of fluorescent *Pseudomonas*	Antifungal activity of fluorescent *Pseudomonas*
0	Bad smell	$129\times10^{-3} = 1.29\times10^{-1}$	N
16	Bad smell	$123\times10^{-3} = 1.23\times10^{-1}$	T
31	Fruity/grape like	$133\times10^{-3} = 1.33\times10^{-1}$	C+
46	Fruity/grape like	$131\times10^{-3} = 1.31\times10^{-1}$	C
61	Fruity/grape like	$127\times10^{-3} = 1.27\times10^{-1}$	C
7 6	Unpleasant	$133\times10^{-3} = 1.33\times10^{-1}$	T
91	Bad smell	$140\times10^{-3} = 1.40\times10^{-1}$	C++
106	Bad smell	$127\times10^{-3} = 1.27\times10^{-1}$	C
121	Bad smell	$126\times10^{-3} = 1.26\times10^{-1}$	T

Colony odor description was done by smelling the plate through the nose and with great care not to inhale the bacteria to avoid bacterial infection, bacterial density count was done by the standard plate count method which consists diluting a sample with serial saline diluents until the bacteria are diluted enough to count accurately, antifungal activity was described qualitatively as indicated below in the table by observing the plates with the naked eye, N: no halo, T: Turbid halo, C: Clear halo, C+: Very clear halo, C++: Very much clear halo, results indicate that at the crop age of 31 days after planting, colony odor, density and antifungal activity of fluorescent *Pseudomonas* was found to be fruity/grape-like, $133\times10^{-3} = 1.33\times10^{-1}$ and C+, respectively for the samples taken from the crop *Vicia faba* L., when the crop age 91 days after planting, odor, density and antifungal activity the bacterium (fluorescent *Pseudomonas*) was found to be bad smell, $140\times10^{-3} = 1.40\times10^{-1}$ and C++, respectively for the same crop sample

Table 3: Morphological characterization of the colony (color, shape and structure) of fluorescent *Pseudomonas* sampled at different developmental stages for the crop *Zea mays* L.

Maize (*Zea mays* L.) age after sowing (days)	Colony color of fluorescent *Pseudomonas*	Colony shape of fluorescent *Pseudomonas*	Colony structure of fluorescent *Pseudomonas*
0	Yellow (white)	Circular	Opaque
16	White mucus	Irregular	Translucent
31	Creamy	Rhizoid	Transparent
46	Creamy	Circular	Opaque
61	Yellow	Irregular	Opaque
76	White mucus	Irregular	Transparent
91	White mucus	Circular	Opaque
106	White	Irregular	Translucent
121	White mucus	Irregular	Translucent

This technique was done by the naked eye and dissecting microscope, results indicate that at the crop age of 31 after planting, the color, shape and structure of the colony of fluorescent *Pseudomonas* were found to be creamy, rhizoid and transparent, respectively for the samples taken from the crop *Zea mays* L), when the crop age is 91 days after planting, color, shape and structure of the colony of fluorescent *Pseudomonas* was found to be white mucus, circular and opaque, respectively for the sample from the same crop

Morphological characterization (color, shape and structure) of the colony of fluorescent *Pseudomonas* for the sample from *Zea mays* L.: The same methodologies indicated under (I) for the crop *Vicia faba* L. is also used here for the crop *Zea mays* L. as shown in Table 3.

Odor, density and antifungal activity of fluorescent *Pseudomonas* sampled at different developmental stages of for the sample from *Zea mays* L.: The same methodologies indicated under (II) for the crop *Vicia faba* L. is also used here for the crop *Zea mays* L. as shown in Table 4.

Table 4: Odor, density and antifungal activity of the colony of fluorescent *Pseudomonas* sampled at different developmental stages for the crop *Zea mays* L.

Faba bean (*Vicia faba* L.) age after sowing (days)	Colony odor of fluorescent *Pseudomonas*	Density of fluorescent *Pseudomonas*	Antifungal activity of fluorescent *Pseudomonas*
0	Bad smell	$119\times10^{-3} = 1.19\times10^{-1}$	N
16	Bad smell	$112\times10^{-3} = 1.12\times10^{-1}$	T
31	Fruity/grape like	$123\times10^{-3} = 1.23\times10^{-1}$	C
46	Fruity/grape like	$121\times10^{-3} = 1.21\times10^{-1}$	C
61	Fruity/grape like	$115\times10^{-3} = 1.15\times10^{-1}$	T
76	Fruity/grape like	$120\times10^{-3} = 1.20\times10^{-1}$	C
91	Bad smell	$128\times10^{-3} = 1.28\times10^{-1}$	C
106	Bad smell	$116\times10^{-3} = 1.16\times10^{-1}$	T
121	Bad smell	$115\times10^{-3} = 1.15\times10^{-1}$	N

Colony odor description was done by smelling the plate through the nose and with great care not to inhale the bacteria to avoid bacterial infection, density count was done by the standard plate count method which consists diluting a sample with serial saline diluents until the bacteria are diluted enough to count accurately, antifungal activity was described qualitatively as indicated below in the table by observing the plates with the naked eye, N: No halo, T: Turbid halo, C: Clear halo, C+: Very clear halo, C++: Very much clear halo, results indicate that at the crop age of 31 days after planting, the odor, density and antifungal activity of colony of fluorescent *Pseudomonas* was found to be fruity/grape like, $123\times10^{-3} = 1.23\times10^{-1}$ and C, respectively for the samples taken from *Zea mays* L., when the crop age is 91 days after planting, the odor, density and antifungal activity of colony of fluorescent *Pseudomonas* was found to be bad smell, $128\times10^{-3} = 1.28\times10^{-1}$and C, respectively for the same crop

DISCUSSION

In this completely randomized design study (Fig. 1), the highest density and strongest antifungal activity of fluorescent *Pseudomonas* was registered for the sample from *Vicia faba* L. when the crop age was around 91 days after being planted in a pot (Table 2 and Fig. 3). The result of this experiment (for 0-60 cm soil depth sample) indicates that the density of the bacterium (colony of fluorescent *Pseudomonas*) and its antifungal activity was significantly varying along with the developmental age of both crops (*Vicia faba* L. and *Zea mays* L.).

For instance in the *Vicia faba* L. experiment, the highest density (140×10^{-3}) and antifungal activity (C++, very much clear halo) of the bacterium were registered (Fig. 3) at around 91 days after crops were being planted in a pot (Fig. 2, to the right hand side). This might be because plants/crops/ produce their microbe attracting exudates differently in terms of quantity and quality at a different developmental stage which in turn influences the bacterial density and the antifungal activity of the bacterium (i.e., fluorescent *Pseudomonas*) existing around their root zone. At this particular developmental age (91 days after planting), the morphological characteristics of colony of the bacterium (color, shape, structure and odor) were found to be white mucus, circular, opaque and bad smell, respectively (Table 1 and 2).

Similarly, there was an increase in the density (133×10^{-3} and antifungal activity (C+, very clear halo) at around 31 days after the crop (*Vicia faba* L.) was being planted (Table 2 and Fig. 3). This might have happened because those crops can attract soil microorganism (bacterium) at the seedling stage (Fig. 2, to the left hand side) not because they secreted too much quality exudates but perhaps vulnerable to soil microbe colonization/pathogen attack. At this developmental age

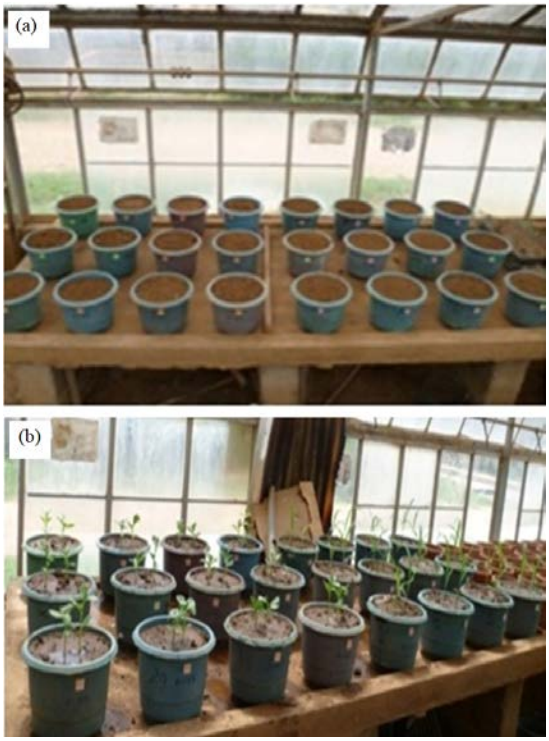

Fig. 1(a-b): Completely randomized design (CRD) under a glass house (a) Before and (b) After seed emergence

(31 days after planting), the morphological characteristics of colony of the bacterium (color, shape, structure and odor) were found to be creamy, rhizoid, transparent and fruity/grape like respectively (Table 3 and 4).

On the other hand for the non-legume crop (*Zea mays* L. experiment), the highest density (128×10^{-3}) and antifungal activity (C, clear halo) of the bacterium were registered at

Fig. 2(a-b): (a) *Vicia faba* L. and (b) *Zea mays* L. grown more under a glass house condition

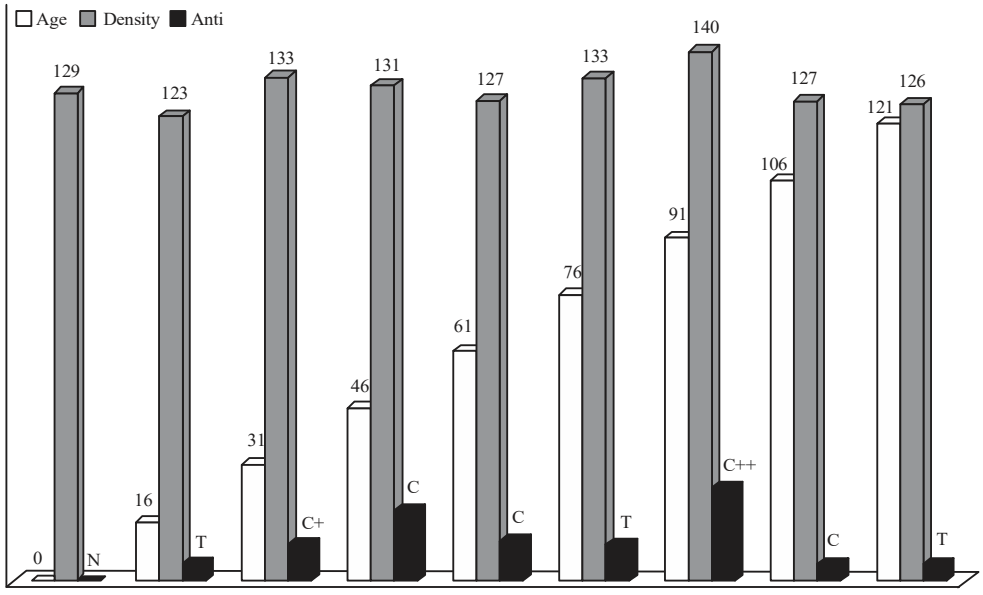

Fig. 3: Effect of plant (*Vicia faba* L.) developmental age on the density and antifungal activity of rhizospheric fluorescent *Pseudomonas*

Age: Age of the crops (days after planting with every 15 days interval assessment by taking a sample from the plant root area), Density: Density of the colony of fluorescent *Pseudomonas*, Anti: Antifungal (anti-Fusarium) activity of fluorescent *Pseudomonas*. N: No halo, T: Turbid halo, C: Clear halo, C+: Very clear halo, C++: Very much clear halo, as it is shown on the above bar graph (Fig. 3), at around 91 days after planting (on the blue bar), bacterial density is highest which is 140×10^{-3} (indicated on the red middle bar) while the antifungal activity is C++ which is very much clear halo (on the light green bar)

around 91 days after crops were being planted in a pot (Table 4 and Fig. 5). Compared to *V. faba* L., the density and antifungal activity for the sample from *Zea mays* L. is less. This might be due to the difference in the crop biology (legume vs. non legume) and other unknown factors which indirectly determines the quantity and quality of the exudate so that influencing the microbial community around the plant root zone[27]. In addition to this, it is also reported that plants recruit different microbes even in different zones of a single root with yet unknown mechanism[28].

Likewise, there was an increase in the density (123×10^{-3} and antifungal activity (C, clear halo) at around 31 days after the crop (*Zea mays* L.) was being planted (Table 4 and Fig. 5). This might be due to similar or different scenarios indicated for *V. faba* L. At this particular developmental age (31 days after planting), the morphological characteristics of colony of the bacterium (color, shape, structure and odor) were found to be creamy, rhizoid, transparent (Table 3) and fruity/grape like (Table 4), respectively which is similar with *V. faba* L.

For the controls where crops were not planted (instead just the dried stick was put at the center of the pots) no

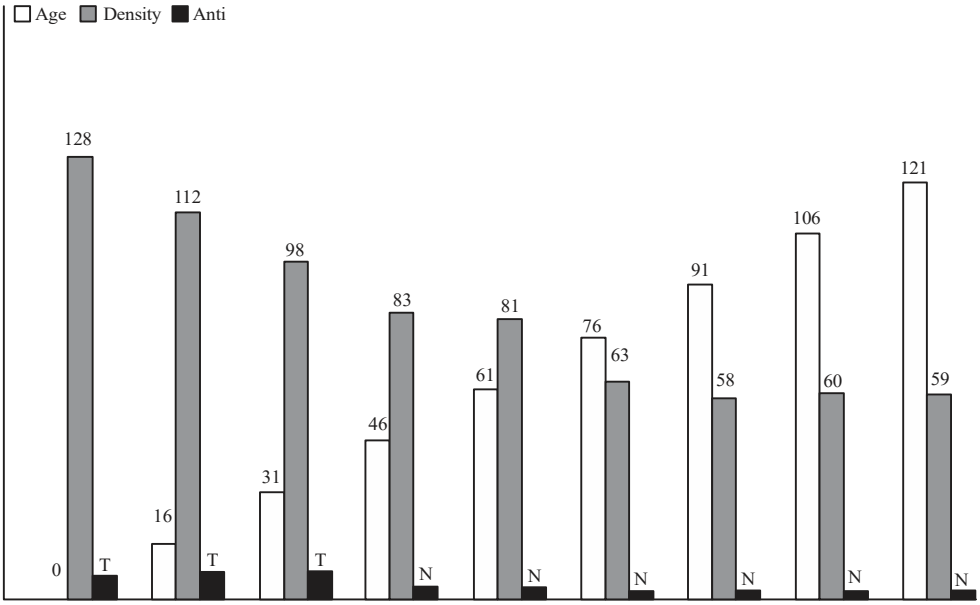

Fig. 4: Control (without planting the crops in a pot) for analysis of effect of plant developmental stage on the density and antifungal activity of fluorescent *Pseudomonas*

Age: 121days with every 15 days interval assessment by taking a sample from the dried stick which was put at the center of the pot, Density: Density of the colony of fluorescent *Pseudomonas*, Anti: Antifungal (anti *Fusarium*) activity of fluorescent *Pseudomonas*, N: No halo, T: Turbid halo, as it is shown on the above bar graph (Fig. 4), density of the fluorescent *Pseudomonas* was declined until 76 days and then almost remained the same (the red bar), at the same time, the antifungal activity of the bacterium was diminished (from turbid to no halo, indicated on the light green bar)

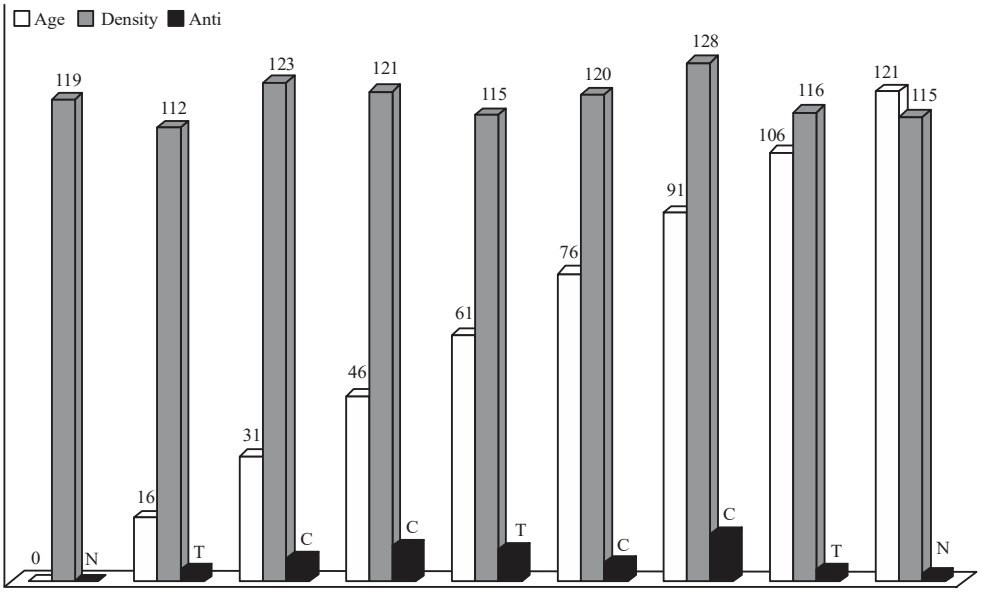

Fig. 5: Effect of plant (*Zea mays* L.) developmental stage on the density and antifungal activity of rhizospheric fluorescent *Pseudomonas*

Age: Age of the crops (days after planting with every 15 days interval assessment by taking a sample from the plant root area), Density: Density of the colony of fluorescent *Pseudomonas*, Anti: Antifungal (anti-*Fusarium*) activity of fluorescent *Pseudomonas*, N: No halo, T: Turbid halo, C: Clear halo, as it is shown on the above bar graph (Fig. 5), at around 91 days after planting (on the blue bar), bacterial density is highest which is 128×10^{-3} (indicated on the red middle bar) while the antifungal activity is C which is halo (on the light green bar)

significant variation was seen for the morphological characteristics of the colony of the fluorescent *Pseudomonas* but its density was declined until 76 days and then almost remained the same (Fig. 4). At the same time, when the days were increased, the antifungal activity of the bacterium was diminished from turbid to no halo (indicated on the light green bar of Fig. 4).

This critical plant developmental age (in this case referred as 91 and 31 days after planting), is only specific to *Vicia faba* L. or *Zea mays* L. considered in the experiment but not for other crops as this age can vary depending on the life cycle of each plant or even varieties of the same crop.

Limitation and suggestion: It is very difficult to correlate our study with other previously conducted experiment since there are no or perhaps very limited research reports that directly configure the relationship between the plant age and the antifungal activities of the bacteria surrounding the plant root.

CONCLUSION

This finding indicated that the highest density of fluorescent *Pseudomonas* was registered from the sample of legume crop *Vicia faba* L., when the crop age was around 91 days after being planted in a pot. The strongest antifungal activity (C++, very clear halo) was seen from the sample of the same crop at this particular stage (91 days after planting). In this experiment, this particular stage for sampling was the developmental stage where both crops were not flowered entirely but almost were ready for it. Therefore considering the plant developmental stage and nature (biology) of the plant during sampling is relevant in the process of isolating and identifying potential bacterial bio-control agent.

SIGNIFICANCE STATEMENT

This study discovers the possible sampling time (from plant developmental stage point of view) to be considered which can be beneficial for researchers on the bio-control field. This study will help the researcher to uncover the critical area of bio-control agent inconsistence field performance that many researchers were not able to explore. Thus, a new theory on consideration of plant age during sampling for screening bio-control agent and possibly other combinations (season of sampling), may be arrived at.

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