Contribution of Pink Pigmented Facultative Methylotrophic Bacteria in Promoting Antioxidant Enzymes, Growth and Yield of Snap Bean

H.G. Abd El-Gawad, M.F. M Ibrahim, A.A. Abd El-Hafez and A. Abou El-Yazied

Abstract: Two field experiments were carried out during the seasons of 2013 and 2014 to investigate the effect of pink pigmented facultative methylotrophic (PPFM) bacteria on the antioxidant enzymes, growth and yield of snap bean plants. The PPFM were isolated from different plants: cotton, datura, snap bean, castor oil and peanut plants. Isolates were compared based on their productivity of indol-acetic acid and cytokinins. Isolate No. 27 was selected due to its high production of both growth hormones and used for plant treatment. The treatments included control (distilled water), foliar application of PPFM, methanol (MeOH) at 5% and the combination of PPFM + MeOH at 5%. Results indicated that spraying the plants with PPFM individually or combined with methanol changed the level of antioxidant enzymes including polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD). Also, the lipid peroxidation as indicated by malondialdehyde (MDA) and morphological studies by scanning electron microscope (SEM) were examined. The PPFM individually achieved the highest significant increases in the number of leaves per plant, average leaf area, haulm fresh weight, leaf chlorophyll, pod number and yield per plant and Feddan in the two seasons compared to the other studied treatments. Moreover, PPFM individually improved the pods quality by increasing their concentrations from amino acids, protein, total sugars and ascorbic acid. Correlation analysis indicated that APX followed by POD as affected by the treatment of PPFM individually related positively to snap bean yield plant$^{-1}$ while, catalase followed PPO affected inversely this trait in both seasons. The SOD was unstable and may not be related to the quantity of yield.

Key words: Antioxidant enzymes · Growth · PPFM · Snap bean · Yield

INTRODUCTION

Snap beans are important protein sources for many developing countries. Also, it is characterized as nearly perfect foods by nutritionists, due to their high content of minerals, vitamins, fibers, complex carbohydrates and other important dietary necessities [1-2].

Pink pigmented facultative methylotrophic bacteria (PPFM), ubiquitous in nature and frequently reported on various plant species, are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves [3]. They are capable of growing on $C_1$ compounds such as formate, formaldehyde and methanol in addition to $C_2$-$C_4$ compounds [4-5]. Moreover, they are able to produce plant growth regulators such as cytokinins and auxins [6-8] which affect plant growth and different physiological processes. The PPFM can also, induce systemic resistance against diseases [9] and degrade a widely range of highly toxic compounds and tolerate heavy metals [10]. Methanol is considered a natural product of plant metabolism, all plant tissues emit methanol [11], especially during early stages of leaf expansion [12]. Some of this methanol is rapidly oxidized in the presence of light to water and CO$_2$ [13]. Generation of CO$_2$ from methanol can also occur by PPFM [7, 14]. Increasing CO$_2$ concentration inside stomata led to accelerate the rate of photosynthesis and decrease the rate of photorespiration in $C_1$ plants [15] because the competition between CO$_2$ and O$_2$ for RuBisco enzyme (the enzyme responsible for reducing CO$_2$ and synthesis of carbohydrates during photosynthesis in $C_3$ plants).
Altering the activity of photosynthesis or respiration is always concomitant with changing in the level of reactive oxygen species (ROS) in plant tissues [16]. ROS are highly reactive and toxic molecules that can affect negatively plant growth and yield and cause damages to proteins, lipids, carbohydrates and DNA. These molecules produced in both unstressed and stressed cells [17]. However, under unstressed conditions, the level of these molecules is in balance because they are scavenged by various antioxidative defense mechanisms [18]. Antioxidant enzymes including polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) are considered the most important protectants in this respect.

Many studies proved the positive effect of PPFM on the growth and yield of plants. Holland [19] reported that the invention provided a method for increasing productivity of a plant by spraying PPFM on a plant. Also, the PPFM could promote red pepper plant growth and yield [20]. Inoculation with PPFM had significant positive effects on growth and yield of several plant species, i.e. soybean [21] and peanut [22]. Sadeghi-Shoae et al. [23] found that PPFM improved plant growth and development of mung bean. The PPFM accelerate growth and yield of sugarcane. It increased specific leaf area, plant height, number of internodes and cane yield. These effects might be mediated by the production or synthesis of plant hormones [24]. The PPFM enhanced seed germination, seedling length and vigor of wheat [3]. Also, Raja and Sundaram [25] found that PPFM inoculation increased seedling vigor, dry matter production and yield of cotton. In this respect, inoculation of PPFM along with chemical fertilizers resulted significant increase in growth parameters of cotton [26]. Radhika et al. [27] concluded that application of PPFM increased the productivity as well as quality parameters of baby corn. Madhaiyan et al. [24] revealed that inoculation of PPFM resulted in significant increase in sugar cane plant growth, cane yield and sugar quality.

Two objectives can be suggested to this study, firstly, assessment the effect of pink pigmented facultative methylotrophic bacteria on antioxidant enzymes, growth and yield of snap bean (Phaseolus vulgaris L.) in clay loam soil. The chemical analyses of the experimental soil are shown in Table (1).

The Experimental Layout: Seeds of snap bean “Bronco” cultivar were sown on the 2nd and 3rd of March 2013 and 2014 seasons, respectively. The area of the experimental plot was 14 m² consisted of five rows; each row was 4 m length and 0.7 m width. The plant distance was 7 cm apart on one side. Calcium super-phosphate (15 % P₂O₅) at the rate of 300 kg / fed was banded on rows at two times, the first (200 kg) was added during soil preparation and the second (100 kg) was carried out in the flowering period. Ammonium nitrate (33% N), at 250 kg / fed., was applied as soil application in two portions, the first after two weeks from sowing at the first irrigation (Mohaya) and the second after one month from the first addition. Potassium sulphate (48 % K₂O) was applied at a rate of 100 kg K₂O fed⁻1 at two times, the first took place two weeks after sowing at the first irrigation, whereas, the second was added one month later. Cultural management, disease and pest control programs were followed according to the recommendations of the Egyptian Ministry of Agriculture.

Preparation of PPFM

Green leaf Samples: For isolation of methylobacteria, samples of green leaves were collected from different plants, Gossypium herbaceum (cotton), Phaseolus vulgaris (snap bean), Vicia faba (broad bean), Datura inoxia. All samples were collected from Qalyubia, Beheira and Giza governorates.

Media Used: Ammonium mineral salts agar medium (AMS) was used for isolation and purification of PPFM [28]. Filtered methanol was added to sterilized medium at 0.5 % concentration. Minimum mineral medium M72 [29]. This medium was used for methanol utilization test. Filtered methanol was added to sterilized medium at different concentrations: 0.5, 1, 1.5 or 2 %. K medium [30] was used for determination of cytokinins. Filtered methanol was added to sterilized medium at 0.5 % concentration. The DSM 125 medium was used for determination of IAA [31].

Isolation and Purification of PPFM: The PPFM were isolated by leaf print method according to Holland et al. [32], fully expanded leaves were collected and samples were handled aseptically, representative samples were collected to fit into 10 cm diameter Petri dishes, then firmly
Table 1: *Chemical properties of the experimental soil in the two seasons (2013 and 2014)

<table>
<thead>
<tr>
<th>Season</th>
<th>N</th>
<th>P</th>
<th>K</th>
<th>pH</th>
<th>Fe</th>
<th>B</th>
<th>Zn</th>
</tr>
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<tbody>
<tr>
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<td>45</td>
<td>88</td>
<td>457</td>
<td>7.30</td>
<td>5.41</td>
<td>4.44</td>
<td>3.50</td>
</tr>
<tr>
<td>2014</td>
<td>74</td>
<td>102</td>
<td>370</td>
<td>7.49</td>
<td>4.76</td>
<td>4.20</td>
<td>3.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Soluble anions (meq/L.)</th>
<th>Soluble cations (meq/L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCO₃ %</td>
<td>E.C dS/m</td>
</tr>
<tr>
<td>2013</td>
<td>1.92</td>
</tr>
<tr>
<td>2014</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* Central Soil and Plant Analysis Laboratory, Fac. Agric., Ain Shams University, Cairo, Egypt

pressed onto the surface of AMS solid medium and incubated at 28°C for 3-5 days. Small PPFM individual colonies were selected and sub-cultured on AMS agar medium several times. Pure colonies were selected and sub-cultured on slants of the same medium. Incubation was carried out at 28°C for 3-5 days. The pure colonies were kept at 4°C.

**Growth of PPFM Isolates as Affected by Methanol Concentration:** Test tubes containing 5 ml of sterilized minimum mineral medium M72 were prepared. This medium was supplemented with different concentrations (0.5, 1, 1.5 and 2%) of methanol (sterilized by filtration). Then individually inoculated with standard inocula of the proper isolates and incubated at 28°C for 3-5 days. Growth was colorimetrically determined by measuring optical density at 620 nm [33].

**Quantification of Indole Acetic Acid (IAA):** Isolates of PPFM were grown in minimal broth medium (DSM 125) in the presence of the auxin precursor (tryptophan, 1 mM/L). The inoculated flasks were incubated on the rotary shaker (150 rpm) at 25°C for 4 days in dark. The IAA was quantified, using the colorimetric technique by Salkowski reagent as described by [34]. After removing the cells by centrifugation at 10000 x g for 30 min, the culture liquid was mixed 1:1 (v/v) with salkowski reagent (12g/L FeCl₃, 7.9 M H₂SO₄) and incubated for 30 min in dark. Thereafter, the optical density was measured using a spectrophotometer at wavelength 530 nm. Amounts of IAA were calculated according to standard curve of IAA.

**Cytokinin Determination:** The isolates of PPFM were grown in K medium with 0.5% methanol [30]. Cells were harvested by centrifugation at 10000 x g for 30 min and the supernatant was used for analysis of cytokinins. The technique of Fletcher and McCullagh [35] was adopted. Cucumber (*Cucumis sativus* L.) and Beta Alfa seeds were germinated in Petri dishes in dark at 28°C. After 6 days, the cotyledons were excised in dim green light and placed in 5 cm Petri dishes (10 cotyledons in each) containing 6 ml of the supernatant of each tested culture. The dishes were returned back to the dark at 28°C for 14 h then moved into fluorescent light with an intensity of 220 ft.c. After 3 h, the chlorophyll from 10 cotyledons was extracted with cold acetone, brought up to a volume of 10 ml and centrifuged at 2500 x g for 10 min. The chlorophyll contents were determined by measuring their absorbance at 665 nm. Amounts of cytokinins were calculated based on standard curve of cytokinins.

**Identification of the Selected PPFM Isolates:** The PPFM isolate highest in cytokinin and IAA production was identified according to Bergey’s Manual of Systematic Bacteriology [36] as follows:

- **Morphological characteristics.** Pure colonies were microscopically examined according to Barrow and Feltham [37] to determined Gram reaction and cell shape, while motility and growth were examined in liquid culture. Cultural characteristics were determined on Ammonium mineral salts (AMS) agar medium [28] after incubation for 3 days at 28°C using binocular microscope.
- **Physiological characteristics.** Catalase, urease and oxidase tester and PHB detection, starch hydrolysis, carbon source utilization and growth on peptone rich nutrient agar were carried out according to Green and Bousfield [38, 39] and Jenkins and Jones [40].

**The Experimental Treatments and Design:** The experiment included the following treatments:

- Control (distilled water).
- Foliar application of PPFM.
- Foliar application of MeOH at 5%.
- Foliar application of PPFM and MeOH at 5%.
Plants were sprayed with the used foliar application of PPFM two times during the growing season. The first spray was followed after 25 days from planting date; the second spray was followed after 21 days from the first spray. The spray solutions were maintained just to cover completely the plant foliage. The experiments were arranged in a randomized complete block design with four replicates.

Parameters Measured

**Determination of Lipid Peroxidation:** In order to determine the level of lipid peroxidation, the method described by Heath and Packer [41] was followed. The level of lipid peroxidation was determined in terms of malondialdehyde (MDA) by using thiobarbituric acid (TBA) test. Frozen tissues were homogenized in 0.1% (w/v) trichloroacetic acid (TCA). The extraction ratio was 10 ml for each one gram of plant tissues. The homogenate was centrifuged at 5000 rpm for 10 min. The reaction mixture contained 1 ml from the supernatant and 4 ml 0.5% (w/v) TBA dissolved in 20% (w/v) TCA. The mixture was heated in boiling water for 30 min then the mixture was cooled at room temperature and centrifuged at 5000 rpm for 15 min. The absorbance of the supernatant was measured at 535 nm using spectrophotometer. The concentration was calculated by using the extinction coefficient (ε = 155 mM<sup>-1</sup>cm<sup>-1</sup>). The concentration of MDA was expressed as nmol MDA.g<sup>-1</sup> FW.

**Enzyme Assays:** Enzyme crude extract was prepared as follows: Leaf tissue (500 mg) was homogenized in 4 ml 0.1M sodium phosphate buffer (pH 7.0) containing 1% (w:v) polyvinylpyrrolidion (PVP) and 0.1mM EDTA. The homogenate was centrifuged at 15000 xg for 15 min and supernatant obtained was used as enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0–4°C. Protein content in the extract was determined according to the method of Bradford [42] and utilizing bovine serum albumin as standard. Polyphenol oxidase (PPO, EC 1.14.18.1) activity was measured according to Oktay et al. [43]. The reaction mixture consisted of 100 µl crude enzyme, 600 µl catechol and 2.3 ml phosphate buffer (0.1 M, pH 6.5). The absorbance at 420 nm was recorded at zero time and after 1 min using spectrophotometer (UV-visible-160A, Shimadzu, Japan). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min. The enzyme activity was expressed as unit.mg<sup>-1</sup> protein. The activity of peroxidase, (POD, EC 1.11.1.7) was assayed according to the method of Dias and Costa [44] with some modifications. The following reaction mixture (3 ml) consisted of 10 mM sodium phosphate buffer, pH 7.0, 50µL enzyme extract and guaiacol 1% (v:v) aqueous solution. The reaction was started by adding H<sub>O</sub><sub>2</sub> at 100 mM. The changes of optical density at 470 nm were recorded in a spectrophotometer. Unit of enzyme (IU) equal 0.01Å OD. Min<sup>-1</sup>. The specific activity expressed as IU.min<sup>-1</sup>.mg<sup>-1</sup> protein. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined according to Nakano and Asada [45]. The decrease of absorbance at 290 nm was monitored in 10–180 s intervals. Catalase (CAT, EC 1.11.1.6) activity was determined according to the method of Chance and Maehly [46] as modified by Cakmak et al. [47]. ACT activity was measured by monitoring the decrease in absorbance at 240 nm following the decomposition of H<sub>O</sub><sub>2</sub> for 1 min using spectrophotometer. The reaction mixture with a total volume of 3 ml contained 15 mM H<sub>O</sub><sub>2</sub> in 50 mM phosphate buffer (pH=7). The reaction was initiated by adding 50 µl crude enzyme. The activity was calculated from extinction coefficient (ε = 40 mM<sup>-1</sup> cm<sup>-1</sup>) for H<sub>O</sub><sub>2</sub>. One unit of enzyme activity was defined as the decomposition of 1 µmol of H<sub>O</sub><sub>2</sub> per minute. CAT activity was expressed as unit.mg<sup>-1</sup> protein. Superoxide dismutase (SOD, EC 1.15.1.1) assay was based on the method described by Beyer and Fridovich [48]. The reaction mixture with a total volume of 3 ml contained 150 µM H<sub>O</sub><sub>2</sub> in 50 mM phosphate buffer (pH=7), 75 µM NBT, 13 mM L-methionine, 0.1 mM EDTA and 0.5 mM riboflavin. The reaction was initiated by the addition of riboflavin then the reaction mixture was illuminated for 20 min with 20 W fluorescent lamp. One unit of enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition in the rate of nitro blue tetrazolium (NBT) reduction at 560 nm using spectrophotometer. The enzyme activity was expressed as unit.mg<sup>-1</sup> protein.

**Scanning Electron Microscope (SEM):** Fresh materials were fixed in 3% glutaraldehyde for 24 h at 4°C [49]. The specimens were dehydrated using ascending concentration of ethanol; critical point dried and finally coated with gold. The morphological examination was achieved through a Jeol Scanning Electron Microscope (JES-T330A) equipped with image recording and processing system (Sem Afor), Central Lab, Faculty of Agriculture, Ain Shams University.

**Vegetative Characteristics:** Ten plants were chosen at random from four replications (from the inner rows) after 50 days from sowing to study number of leaves per plant. Average leaf area using the fourth full expanded mature
leaf from the plant top. Haulm fresh weight per plant was recorded. Leaf chlorophyll reading (SPAD) was determined using the fourth expanded full mature upper leaf of 21 plants in the middle row per plot. A digital chlorophyll meter, Minolta SPAD-502, (Minolta Company, Japan) was used. The SPAD readings were used as relative values for chlorophyll content.

**Yield and Quality Characteristics:** Number of pods per plant, pod yield per plant and Feddan were recorded from ten plants. Free amino acids (mg/g FW.) were determined according to Hamilton and Van Slyke [50], fresh plant material (0.5g) was homogenized in 5 ml phosphate buffer (pH = 7.0) and centrifuged then one ml of each sample extract was treated with 1ml of 10% pyridine and 1 ml of 2% ninhydrine solution, the optical densities of these colored solutions were then read at 570 nm. Total protein percentage in the extract was determined according to the method of Lowry et al. [51] and utilizing bovine serum albumin as standard. Total sugars (mg/g FW.) and L ascorbic acid (L.A.A.) (mg/100 g FW.) concentration in pods were measured according to A.O.A.C. [52].

**Statistical Analysis:** Data of the two seasons were arranged and statistically analyzed using Mstatic (M.S.) software. The comparisons among means of the different treatments were determined as illustrated by Snedecor and Cochran [53]. Standard error of the means (S.E) was calculated and LSD’s test based on a probability of $P \leq 0.05$ was used to determine significant differences between means.

**RESULTS AND DISCUSSION**

**Isolation and Purification of PPFM:** Data in Table (2) illustrated the number of PPFM isolates obtained from cotton, datura, snap bean, castor oil plant and peanut plants from three different locations of Egypt. The obtained isolates were tested for their abilities to produce IAA and cytokinin. Table (3) show that isolate No. 19 was the best in IAA production while for cytokinin, isolate No. 27 was the highest one, which was also second best in IAA production. Therefore, the isolate No. 27 was selected for the following experiments. Identification results showed that the selected PPFM isolate is mostly related to the genus *Methylobacterium*. Identification methods used were not sufficient to identify the species.

**Lipid Peroxidation:** Data in Figure (1) illustrates that malondialdehyde (MDA) concentration in leaves increased by all treatments compared to controls in both seasons. The highest significant ($P \leq 0.05$) results were achieved by exposure to MeOH (230, 357%) as individual followed by its combination with PPFM (181, 278%) over controls in both seasons respectively. The MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury [54]. It is a highly reactive molecule and reacts rapidly with DNA and proteins, causing modification to their structures [55]. In this study, the high increases of MDA concentration by the methanol in the treated plants could be attributed to the increase in the free radicals generated during the metabolism of alcohol which can react with proteins and lipids changing their structure and functions [56] or for the ability of MeOH as a solvent for lipids bilayer of cell membranes according to its concentration and the type of plant tissues. On the other hand, the insignificant increment in the MDA concentrations by the individual treatment of PPFM may be necessary to improve the plant

### Table 2: Pink pigmented facultative methylotrophic bacteria isolated from the phyllosphere of different plants distributed among three governorates in Egypt

<table>
<thead>
<tr>
<th>Site of cultivation</th>
<th>Plant species</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qalyubia</td>
<td>Cotton</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Datura</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Kidney bean</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Castor oil plant</td>
<td>4</td>
</tr>
<tr>
<td>Giza</td>
<td>Cotton</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Peanut</td>
<td>3</td>
</tr>
<tr>
<td>Beheira</td>
<td>Cotton</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Peanut</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 3: Assessment of IAA and cytokinins production by PPFM isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>IAA (µg/ml)</th>
<th>Cytokinins (µg/ml)</th>
<th>Isolate No.</th>
<th>IAA (µg/ml)</th>
<th>Cytokinins (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.964</td>
<td>1.027</td>
<td>16</td>
<td>.960</td>
<td>0.935</td>
</tr>
<tr>
<td>2</td>
<td>.952</td>
<td>1.333</td>
<td>17</td>
<td>.785</td>
<td>0.877</td>
</tr>
<tr>
<td>3</td>
<td>.889</td>
<td>1.183</td>
<td>18</td>
<td>.955</td>
<td>1.182</td>
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<tr>
<td>4</td>
<td>.878</td>
<td>1.206</td>
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<tr>
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<td>1.057</td>
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<tr>
<td>6</td>
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<td>0.993</td>
<td>21</td>
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<td>15</td>
<td>.973</td>
<td>0.948</td>
<td>30</td>
<td>0.952</td>
<td>1.257</td>
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</tbody>
</table>
Fig. 1: Effect of foliar application of pink pigmented facultative methylotrophic (PPFM) bacteria, methanol (MeOH) and their combination on the lipid peroxidation (malondialdehyde=MDA concentration) in the leaf tissues of snap bean plants.

Fig. 2: Effect of foliar application of methylotrophic bacteria (PPFM), methanol (MeOH) and their combination in the activity of some antioxidant enzymes in leaf tissues of snap bean plants.
growth (Figure 5). This result could be related to the fact that it was stated by more than evidence that MDA exists also in healthy tissues. It was found concentrated in meristematic cells (cell division region) of the Arabidopsis seedlings and associated with cell division which led to enhance plant growth [57, 58].

**Antioxidant Enzymes:** Plants possess very efficient antioxidant defense systems (enzymatic and non-enzymatic) which work together to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS [16]. Among the antioxidant enzymes: polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) were determined.

Data presented in Figure 2 indicate that the activity of polyphenol oxidase (PPO) in the leaf tissue of snap bean was decreased significantly (P ≤ 0.05) by the treatment of MeOH as individual (350.7, 369.6 unit. mg⁻¹ protein) or combined with PPFM (364.4, 356.1 unit. mg⁻¹ protein) compared to the controls (436.9, 456.3 unit. mg⁻¹ protein) in both seasons, respectively. On the other hand, PPFM as individual treatment preserved the PPO activity at the same level of significance with controls in the two seasons. The PPO has been implicated in several physiological processes including plant defense against pathogens and insects, the Mehler reaction, photoreduction of molecular oxygen by PSI and regulation of plastidic oxygen levels [59]. The decreases in the level of PPO in the leaves of snap bean might be due to its susceptibility to the treatment with MeOH (Figure 4 c) which led to change in the gene expression. These results are in agreement with Downie et al. [60] who found that exposure to methanol affects the expression of hundreds of genes in *Arabidopsis thaliana* which are responsible for multiple signaling pathways.

Concerning peroxidase (POD), it was obvious that PPFM as individual improved the activity of POD, but these increases were insignificant compared to the controls in the two seasons. An opposite trend was detected when plants were subjected to MeOH individually. Also, it was observed that there were significant (P ≤ 0.05) differences between the individual treatments of PPFM (2534, 2190 unit. mg⁻¹ protein) and MeOH (1961, 1483 unit. mg⁻¹ protein) in both seasons respectively. Peroxidase (POD) constitutes a class of heme-containing enzymes ubiquitously present in prokaryotic and eukaryotic organisms. This enzyme catalyzes the dehydrogenation of structurally diverse phenolic and endolic substances by H₂O₂ and is thus often regarded as an antioxidant enzyme, protecting cells from the destructive influence of H₂O₂ and derived oxygen species [61]. The PPFM are capable to produce auxin [7]. Therefore, enhancing the activity of POD by PPFM as individual treatment could be attributed indirectly to its ability to increase the concentration of endolic substances which act as substrates for POD.

The behavior of ascorbate peroxidase (APX) was similar to POD. Remarkable increments were observed in the activity of APX over the control by the treatment of PPFM individually in both seasons (19.85, 23.67 Unit. mg⁻¹ protein), respectively. The highest significant (P ≤ 0.05) increase reached about 2.25 fold over control was achieved in the second season. APX catalyzes scavenging of H₂O₂ utilizing ascorbic acid as an electron donor [16]. In addition to auxin; PPFM are capable to produce cytokinins (CKs) and vitamin B12 [62, 63]. All of these stimulators may increase the biosynthesis pathway for ascorbic acid which is considered the substrate for APX. Furthermore, CKs play a role in the regulation of hydrogen peroxide (H₂O₂) accumulation [64].

Catalase (CAT) is present in the peroxisomes of nearly all aerobic cells and virtually absent from chloroplast [65]. It can protect the cell from H₂O₂ by catalyzing its decomposition into O₂ and H₂O [66]. In the current investigation, all foliar treatments except PPFM + MeOH in the second season reduced the activity of CAT comparing with controls in both seasons, MeOH gave the lowest decreases (0.079, 0.091 Unit. mg⁻¹ protein) in this respect. The coordinate function and balance between antioxidant enzyme activities such as POD, APX and CAT is crucial for conserving steady state levels of H₂O₂. Therefore, the decreases in CAT activity which obtained by the treatment of PPFM or MeOH may be attributed to the integration between POD and / or APX from side and CAT from the other one. Such this integration could be suggested to compensate the change in the activity for each other.

As for superoxide dismutase (SOD), it was obvious that all treatments decreased significantly the activity of SOD with the exception of PPFM as individual in the first season. The minimum decreases were given by the treatment of MeOH as individual (6.36, 6.08 unit. mg⁻¹ protein) compared to the controls in both seasons. The SOD, among antioxidant enzymes, represents the first line of defense as it catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide and molecular oxygen [67]. Therefore, induction of SOD may reduce the cellular damage caused by oxidative stress.
Fig. 3: SEM photographs showing the epidermal cells on the upper surface (adaxial side) of snap bean leaf after foliar application of pink pigmented facultative methylotrophic (PPFM) bacteria individually and combined with methanol (MeOH). The white arrows refer to the stomata associated with PPFM on and inside the tiny pores. It is clear that PPFM are capable to grow on MeOH.

Fig. 4: SEM photographs showing the effect of foliar application by distilled water as control (A), pink pigmented facultative methylotrophic (PPFM) bacteria (B), methanol (MeOH) (C) and their combinations (D) on the morphological structure of the epidermal cells on the upper surface (adaxial side) of snap bean leaf. It can be observed that the leaf tissue has hypersensitivity to the treatment by MeOH especially as individual, whereas the combined treatment with PPFM reduced this effect.
Morphological Studies by SEM: Morphological studies by SEM (Figure 3) showed that PPFM are localized and concentrated in and around the stomata. Also, there was increase in the number of PPFM when applied in combination with MeOH. This result may be attributed to the importance of MeOH for PPFM to grow.

The observations in Figure 4 revealed that the leaf of snap bean exhibited a hypersensitivity to the treatment by MeOH as individual (Figure 4 c). It appears that there are collateral damages and deformations in the normal structure of epidermal tissue. Two possibilities can be suggested to explain this effect: firstly; MeOH increased the level of ROS according to Skrzydlewska et al. [56] and reduced the most studied antioxidant enzymes (Figure 2) in the leaf tissues. These results led to increase the level of lipid peroxidation (Figure 1) and destroy the structure of the biological membranes; secondly; MeOH has the ability to increase the dehydration which directly results in shrinkage of tissues by withdrawing the water from them. Conversely, as shown in Figure (4 b), PPFM as individual treatment kept the normal structure for the epidermal cells and increased the percentage of opened stomata comparing with control (Figure 4 a). This increase may affect positively on stomatal conductance, photosynthesis and growth (Figure 5) of snap bean whereas, the combination of PPFM with MeOH (Figure 4 d) reduced the harmful effect of MeOH on leaf tissues. This result was also, confirmed by decreasing the level of lipid peroxidation significantly (P≤0.05) in the combined treatment compared to MeOH individually (Figure 1).

Vegetative Growth: Data in Figure (5) indicated that the foliar application of PPFM increased significantly (P≤0.05) number of leaves per plant, average leaf area, haulm fresh weight per plant and leaf chlorophyll reading in the two seasons compared with the other studied treatments. On contrast, the foliar application of methanol individually achieved significant decreases in all previous parameters with exception of number of leaves in the first season whereas, the combination treatment of PPFM + MeOH kept all growth parameter at the same level of significance compared to control in both seasons. The promoting effect of PPFM on vegetative growth was mentioned in many species including red pepper, soybean and mung bean [20, 21, 23]. In the current study, this effect could be explained by that, PPFM have the ability to produce some growth regulators including auxins and cytokinins [7-8]. These results were confirmed in Table (3). Also, our data revealed that MDA
was increased insignificantly by inoculation of PPFM individually compared to untreated plants (Figure 1). These increments may be necessary to improve the vegetative growth because MDA was concentrated in the meristematic cells and concomitant to cell division [57, 58]. On the other hand, the negative effect of methanol as individual treatment could be attributed to the high increases of MDA (Figure 1), decrease most antioxidant enzymes in snap bean leaf tissues (Figure 2) and the deleterious effect of MeOH on the epidermal tissue (Figure 4).

Pods Yield: As shown in Figure (6), inoculation of PPFM increased significantly ($P \leq 0.05$) the pod number, yield per plant and Feddan of snap bean in the two seasons compared to the other studied treatments. These results were emphasized earlier in many species such as soybean, red pepper and peanut [21, 20, 22]. In this study, our data revealed that PPFM had several positive effects at the morphological and biochemical levels of snap bean which can improve the yield and its components. For example, Table (3) showed that PPFM are capable of producing some growth promoters such as auxins and cytokinins.

![Figure 6: Effect of pink pigmented facultative methylotrophic (PPFM) bacteria on yield characters of snap bean plant in the two seasons (2013 and 2014)](image-url)
Fig. 7: Effect of pink pigmented facultative methylotrophic (PPFM) bacteria on bio-chemical components of snap bean pods in the two seasons (2013 and 2014)

Fig. 8: Correlation coefficients between snap bean yield plant\(^{-1}\) and the activity of antioxidant enzymes as affected by the foliar application of pink pigmented facultative methylotrophic bacteria in the two growing seasons of 2013 and 2014

These substances may explain the significant (P ≤ 0.05) increases in all studied vegetative growth parameters (Figure 5) by inoculation of PPFM. The ability of PPFM for producing some growth regulators was confirmed previously in many studies [24, 7, 8]. Besides, Ventorino et al. [68] found that there are several substances enhanced the productivity such as siderophores solubilise phosphate in tomato plants. Another reason can be suggested to explain these influences; PPFM can increase the rate of photosynthesis and reduced photorespiration by elevate the concentration of CO\(_2\) around and inside the stomata pores [15]. This action permits Rubisco enzyme to catalyze the carboxylation and initiate Calvin cycle which is responsible for CO\(_2\) fixation and synthesis of carbohydrates [69].
On the other hand, the foliar spray of methanol individually decreased significantly ($P_{\leq} 0.05$) the pod number, yield per plant and Feddan. These effects could be attributed to increasing the level of lipid peroxidation (Figure 1) and production of ROS without enough coordination in the activity of antioxidant enzymes (Figure 2). The ROS are toxic molecules that affect negatively plant growth and yield and cause damages to proteins, lipids, carbohydrates and DNA. These harmful effects were observed by SEM (Figure 4 c).

**Pods Quality:** Data presented in Figure (7) indicate that the foliar application of PPFM increased free amino acids, protein, total sugars and ascorbic acid in pods. These increases reached the level of significance ($P_{\leq} 0.05$) in free amino acids and ascorbic acid in both seasons and total sugars in the first one, while, protein percentage was insignificant in both seasons. These results could be attributed to the stimulating effect of PPFM on the activity of antioxidant enzymes (Figure 2) and vegetative growth (Figure 6). Also, PPFM can accelerate the rate of photosynthesis and decrease the rate of photorespiration [7]. These responses explain the biochemical changes in pod composition because they affected all physiological and biochemical pathways which related to quality of pods. In strawberry, Verginer et al. [70] reported that PPFM in vitro can increase the production of furanoid compounds, 2,5-dimethyl-4-hydroxy-2H-furanone, which are responsible for strawberry flavor, showing that PPFM can influence fruit quality.

**Correlation Coefficient:** Correlation analysis (Fig. 8) identified that changes in the activity of APX followed by POD were the most important positively related enzymes to snap bean yield plant$^{-1}$ as affected by the foliar application of pink pigmented facultative methylotrophic (PPFM) bacteria in both seasons. On the other hand, CAT and PPO affected inversely this trait while, the behavior of SOD was unstable between the two seasons.

**CONCLUSION**

Based on the present study, it could be concluded that antioxidant enzymes, vegetative growth, pod yield and its quality of snap bean were responded positively to foliar application of pink pigmented facultative methylotrophic bacteria under the experiment conditions.

**REFERENCES**


