Research Article

Gibberellic and Indole Acetic Acids Producing Features of Bacteria from the Genus *Lactobacillus* and their Effect on Plant Development

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Submission Date: 26-11-2021; Revision Date: 14-12-2021; Accepted Date: 19-12-2021

ABSTRACT

Gibberellic acid (GA), also known as gibberellin A3, GA, and GA_3, and indole acetic acid (IAA) are phytohormones that enhance plant growth and are key properties of soil microorganisms, rhizosphere bacteria, micromycetes, and lactic acid bacteria. In this research work, lactic acid-synthesizing bacteria were isolated from a variety of plants and their effects on the growth of wheat coleoptiles were studied. High growth activity of *Lactobacillus* bacteria and the formation of GA and IAA were detected by hyphenated HPLC-MS, and its antifungal activity was studied against some phytopathogenic micromycetes. It should be noted that despite the widespread use of lactic acid bacteria, their effect on plant development, their phytohormone forming features have poorly been studied and now, it becomes a new direction in science.

Key words: *L. plantarum* M, *Bacillus* sp. *L. plantarum* M-1, *L. plantarum*-V, *L. plantarum*-S, *L. plantarum*-G, *L. plantarum*-D, Lactobacillus, Bacteria, Phytohormones.

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INTRODUCTION

In the world, special attention is paid to the cultivation of agricultural crops based on the technology of production of ecologically pure products. This requires increasing the productivity of agricultural crops by reducing or eliminating the use of chemical fertilizers, and the effective use of local biological preparations that do not adversely affect nature and the environment. Another advantage of using environmentally friendly and safe biological agents is that they, increase the germination capacity of seeds, enhance plant growth, accelerate flowering and fruit ripening. These factors are influenced by metabolites produced by microorganisms. Metabolites that stimulate growth also affect the level of crop yields, product quality, increase the amount of proteins, essential amino acids, vitamins, and protect from bacterial and fungal diseases.

Biological preparations are specific nutrients for plants that contain active microorganisms and help seeds to grow. Biological preparations contain microorganisms that also break down organic matter and produce natural phytohormones and vitamins that are easy for plants to assimilate.[1] It is known that the growth and vigorous development of plants is greatly influenced by the activity of microorganisms present in the rhizosphere.[2] Representatives of many species of microorganisms are the most active producers of compounds with several practical valuable biological activity (amino acids, group of vitamins, enzymes, polysaccharides, bacteriocins, antibiotics and phytohormones).[3] Faridah *et al.* Studied Phytohormonic activity of *Lactobacillus* (IAA – 6.175 mg/ml, GA – 2.177 mg/ml), *Azotobacter* (IAA -3.381 mg/ml, GA -1.86 mg/ml), *Streptomyces* (IAA-3.651 mg/ml, GA – 2.575 mg/ml) species included in "MO Plus" and "Paenibacillus polymixa" biological preparations with antagonistic features. Isolates of "MO Plus" biological preparation (IAA-5.619 mg/ml, GA-2.776 mg/ml) and "Paenibacillus polymixa" preparation

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DOI: 10.5530/ajbls.2021.10.91

(IAA -9.508 mg/ml, GA-1.620 mg/ml) were found to synthesize phytohormones.[1] When microorganisms-synthesizing GA was applied to corn (*Zea mays* (L.)), an increase in the activity of phytohormones in the plant and in its ability to resist salinity stress was observed.[4] There are more than 136 species of gibberellins, which have a positive effect on plant growth and development (GA3, GA4, GA7). GA_3 is of great importance in agriculture and industry as it stimulates growth at low concentrations (Î¼g), accelerating flower and seed formation, strongly developing the root system and enhancing seed germination, and has a rapid effect unlike other phytohormones.[5] Application of GA_3 synthesized by microorganisms at a concentration of 150 mg/ml was found to be the most active in the growth of corn hypocotyl and enhance seed germination, activate the initial development phase of the plant, expand the leaf plate surface.[6,7] Indole acetic acid (IAA) production is a key feature of rhizosphere bacteria, which also stimulate plant growth and enhance plant development.[8] IAA is the most important member of the auxins group, being synthesised by gram-negative and gram-positive bacteria.[6,9] When culture fluid of *Rhodobacter sphaeroides*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* bacteria was applied in melons, the roots of the plant developed strongly, chlorophylls increased significantly, and young sprouts grew rapidly. Inoculation with *Rhodobacter sphaeroides* culture fluid affected plant growth, while inoculation with *Lactobacillus plantarum* significantly increased gibberellin level and plant growth due to a decrease in the amount of abscisic acid.[10] The medicinal properties of the plant *Cannabis sativa L.* is reported to have antagonistic activity against phytopathogenic *Staphylococcus aureus* and *Bacillus cereus of ethanolic extracts of* lactic acid bacteria (*Lactobacillus plantarum* KCTC 3107, *L. plantarum* KCTC 3108, *L. brevis* BHN-LAB128, *L. paracasei* BHN-LAB129), with the formation of 13.99 mm and 15.17 mm rings, respectively. which includes ethanolic extracts of lactic acid bacteria (*Lactobacillus plantarum* KCTC 3107, *L. plantarum* KCTC 3108, *L. brevis* BHN-LAB128, *L. paracasei* BHN-LAB129) showed relatively antagonistic activity to phytopathogenic *Staphylococcus aureus*.and *Bacillus cereus*, and was found to form a ring of 13.99 mm and 15.17 mm, respectively.[11] When wild forest plants (Morinda coreia Ham) were treated with microorganisms such as *Lactobacillus plantarum, Lactobacillus pentosus, Saccharomyces cerevisiae*, the amount of microelements B, Mn and Zn was significantly increased during fermentation. It has also been proven that *L. plantarum* is dominant in long-term fermentation, loosing of pathogenic microflora and using as liquid fertilizer in the absence of micronutrients (B, Mn, and Zn) in plants.[12] It can also be used in the disinfection of cereal seeds used in the brewing industry. The use of culture fluid *Lactobacillus plantarum* VTT E-78076 (E76) and *Pediococcus pentosaceus* VTT E-90390 (E390) in the process of wetting barley seeds with water eliminates the development of conditionally pathogenic yeasts, gram-negative bacteria and dangerous pathogens belonging to the genus *Fusarium*.[13,14] Therefore, *L. plantarum* E76 and *P. pentosaceus* E390 bacteria can be used in the effective storage of seeds of cereals and in the production of the products from them.[15,16]

RESEARCH METHODS

Selection of lactic acid bacteria on the basis of GA and IAA formation properties and determination of the growing properties of the selected bacterial strains

Hypocotyl method screening was performed on plant growth characteristics of bacterial strains belonging to *L. plantarum 5, Bacillus.sp. L.plantarum M, L. plantarum-V, L. plantarum-S, L. plantarum-G, L. plantarum-D, Lactobacillus* species. For this, 10 different bacterial strains were grown in liquid DeMannRogosaaSharpe (MRS)[17] nutrient medium for 6 days and the culture fluids were filtered through a 0.22 μm bacteriological filter. The bacterial strains were then grown in DeMannRogosaaSharpe (MRS) (pH 6.2) nutrient media at 37°C for 6 days in a shaker at a rotation rate of 200–220 times per minute. The effect of the filtered culture fluids was observed for 24 hr on 1 cm long colioptiles of wheat and then measured. The effect of culture fluids of the selected bacterial strains on the germination of wheat seeds was also studied.

The culture liquids of the isolated bacterial strains grown in MRS liquid nutrient medium for 6 days, were filtered and diluted in ratios of 1:50, 1: 100, 1: 200, 1: 400, 1: 500 and were inoculated into the plant seeds for 2 hr. In the control variant, instead of the culture fluid, the seeds were treated with water and MRS nutrient medium. 50 plant seeds were taken for each experimental group. The experiment has been performed in three replications. The seeds were grown on a Petri dish in a thermostated condition at 25°C for 48 hr and the germination percentage was determined.[18]

Determination of gibberellic acid content. The isolated lactic acid bacterial strains were grown in MRS medium at a temperature of 37°C in a shaker at 200-220 rotations/min for 6 days. The Muromtsev and Nestyuk method[19] was used to determine GA content in culture fluid of the selected bacterial strains. To do this, 1.0 ml of filtered culture fluid was taken into test-tubes of

15-20 ml to which 1.0 ml of Folin-Chiocalto (100 g Na_2O_4W + 25 g Na_2MoO_4 + 700 ml of water + 50 ml of 85% + H_3PO_4 + 100 ml of conc. HCL + 150 g Li_2SO_4 + 2-3 drops of Br) reagent and HCl were added to acidify it and all mixed well. The reaction mixture was left in a dark place for 40 min. The GA samples turned light and dark green, and the GA content was measured on a SPEKOL 1300 spectrophotometer with a red light filter at a wavelength of 750 nM according to the optical density of the supernatants. The concentration of GA was determined using a graph calibrated according to the standard of GA (Sigma Aldrich, CA, USA).[20]

Determination of indole acetic acid content. The content of IAA was measured for 6 days from the second day of bacterial growth. The Gordon and Weber method[21] was used to determine the content of IAA in the bacterial culture medium. To do this, to 1.0 ml of filtered culture fluid taken into the test tubes of 15-20 ml, 8.0 ml of Salkovsky reagent (mixture of 50 ml 35% solution of H_2SO_4 and 1 ml 0.5 M solution of $FeCl_3$) was added and mixed well. Then, the reaction mixture was left for 30 min. When the samples turned red-pink, the IAA content was measured on a SPEKOL 1300 spectrophotometer according to the optical density of the supernatants through a green light filter at a wavelength of 450 nM. The indole acetic acid concentration was determined using a calibrated graph for pure indole acetic acid (Sigma Aldrich, CA, USA).[22]

Extraction of GA from the culture fluid of selected lactic acid bacteria

After the growth of selected bacterial strains at 37°C in 250 ml of MRS nutrient medium by shaking at 220 rpm/min for 6 days in 500 ml Erlenmeyer flasks, the culture fluid was filtered by a 0.22 µm bacteriological filter. The pH value of the isolated culture fluid was equalized to 2–2.2 with 2 N HCl and the resulting GA was isolated by extraction 3 times in ethyl acetate.[21] The extraction sum of 10 g of ethyl acetate was dried under vacuum conditions (RE100-Pro rotary evaporator) (Luetal., 2014) at 40°C. The obtained GA was subjected to HPLC-MS analyses.

HPLC-MS analysis of GA and IAA

In the detection of GA by high-performance liquid chromatography-mass spectrometry (HPLC-MS), the bacterial strain was filtered through 0.22 µk/ml filter, adjusted to 2-2.2 with 2 N HCl and extracted 3 times with ethyl acetate.[21] The extraction sum of 10 g of ethyl acetate and 15 g of butanol was dried under vacuum conditions (RE100-Pro rotornyy isparitel) (Luetal., 2014) at 40°C (50 rot./ min). For HPLC-MS analyses have been performed on TSQ Quantum Access Max ultra (TSQ) Ultimate 3000 Quaternary Standard (Thermo Fisher Scientific, USA) with tertiary quadrupole mass spectrometry. Chromatographic conditions were selected as follows: Column Hypersil GOLD aQ 100 mm x 2.1 mm, 1.9 µm. Mobile phase: 2 min. H_2O - in 100% isocratic elution, then within 12 min. water/acetonitrile – 0:80%, 1 min. acetonitrile 80%, 2 min. water/acetonitrile - gradient eluent in the ratio 100:0%. The column temperature was set at 35°C. Mass spectrometric conditions for heated-electrospray ionization (HESI-II) were selected as follows:

Spray voltage (V) - for 4000 positive ion monitoring;
Vaporizer temperature (ºC) - 300;
Sheath gas pressure (psi) - 35
Auxiliary gas pressure (psi) - 10;
Capillary temperature (ºC) - 350;
Tube Lens Offset - 80
Collision pressure (mTorr) - 1.5;
Collision energy (eV) - 20.

HPLC-MS/MS analysis were performed in a full scan mode, in the range of 15–1500 m/z.

Statistical Analysis

From the plants of mango (*Mangifera*) regionized in Uzbekistan, medicinal valerian (*Valeriána officinális*), magnolia (*Magnolia*), vaccinium (*Vaccínium uliginósum*), Crássula, lilac (Syrínga), mint (Mentha), dandelion (Taráxacum), lettuce (Lactúca satíva) and juniper (*Juniperus*), 10 bacteria strains belonging to *Lactobacillus* species were isolated and identified by their morphological traits. For the determination of plant growth stimulating features of isolated bacterial strains of *L.plantarum M, Bacillus.sp. L.plantarum M-1, L.plantarum-V, L.plantarum-S* species, a thorough research with experiments has been conducted. In this case, plant growth stimulating features of isolated bacterial strains were studied in wheat coleoptiles by the Hypocotyl method (Figure 1).

The experiment was performed in 5 replicates. In the experimental variants, 10 bacterial strains of the local *Lactobacillus* species isolated from culture fluid and distilled water in control variant were used.

Figure 1: Influence of lactic acid bacteria culture fluid on wheat coleoptiles.

Out of 10 bacteria examined in this research, *L. plantarum 5, Bacillus.sp, L. plantarum M, and L. plantarum L* strains were found to have higher activity to wheat coleoptilli growth. The highest growth activity was observed in the variant in which *L.plantarum 5* bacterial culture fluid was used. A wheat (Tanya) coleoptile with a length of 1.82 mm was observed relative to the control (Figure 2). When wheat seed was inoculated with *Lactobacillus plantarum* bacterial culture fluid, the plant's resistance to external abiotic stresses increases, and the attraction of lactic acid bacteria to nitric oxide increases the plant's resilience to stress.[23-25,19] In the next phase of the study, an experiment was conducted to determine the effect of bacteria on wheat germ germination.

High levels of seed germination and underdevelopment of phytopathogenic microflora were detected when wheat seeds were inoculated with culture fluid of lactic acid bacteria at a concentration of 1/100. The highest germination of seeds was observed in the variant using a concentration of 1/100 of *L. plantarum 5* culture fluid, and 93% germination of seeds was found to have 30% higher than in the control (water) variant and 93% higher germination was noted than in the control (MRS nutrient) medium (Figure 3).

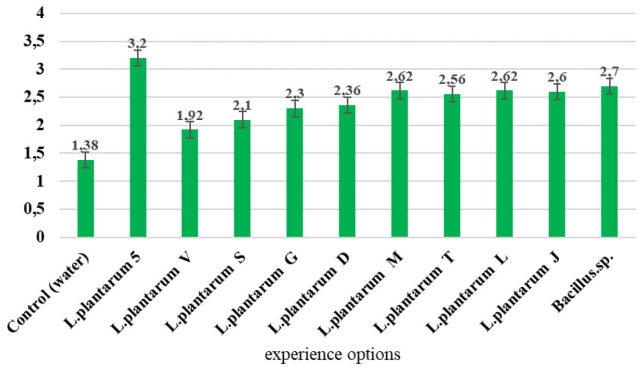

Figure 2: Influence of lactic acid bacteria culture fluid on the growth of wheat coleoptiles.

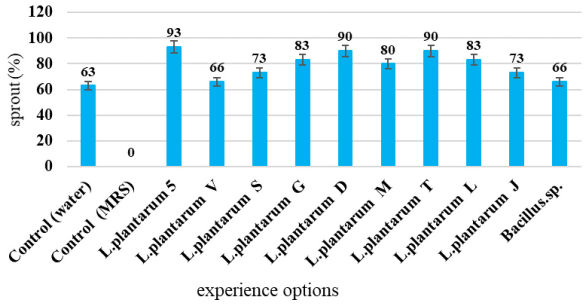

Figure 3: Influence of lactic acid bacterial culture fluids on wheat seed germination.

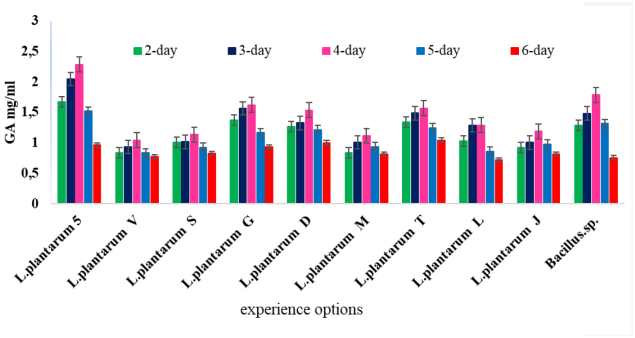

Figure 4: Quantitative analysis of GA synthesis in the culture fluid of lactic acid bacteria (day 6).

During the study, the phytohormone-forming properties of the selected lactic acid bacteria were determined. Quantitative analysis of GA and IAA in the culture fluid of the selected lactic acid bacteria was performed on 1300 -SF, (spectrophotometer).

The highest production of GA in isolated lactic acid bacterial strains was detected on the 4th day of the growth. *L. plantarum V.* bacterial strain culture fluid formed 1.042 mg/ml GA on the 4th day of the growth, *L. plantarum S* formed 1.133 mg/ml GA, *L. plantarum G* 1.623 mg/ml, *L. plantarum D* 1.535 mg/ml, *L. plantarum M* 1.115 mg/ml, *L. plantarum T* 1.567 mg/ml, *L. plantarum L* 1.290 mg/ml, *L. plantarum J* 1.187 mg/ml, *Bacillus. sp.* 1.786 mg/ml. The highest amount of GA was detected in the culture fluid of *L. plantarum 5* bacterial strain and produced 2.286 mg / ml GA on the 4th day of growth (Figure 4). Quantitative analysis of IAA in the culture fluid of selected lactic acid bacteria was determined on 1300 -SF, spectrophotometer.

The highest levels of IAA were detected in selected lactic acid bacterial strains on the 3rd and 4th days of growth.[26] *L. plantarum V.* bacterial strain culture fluid formed 1,142 mg/ml IAA on the 3rd day of growth, while on the 4th day 0,824 mg/ml, *L. plantarum S* 0,452 mg/ml, on the 4th day 0,352 mg/ml, *L. plantarum G* 1,385 mg/ml, on the 4th day 1,135 mg/ml, *L. plantarum D* 1,135 mg/ml, while on the 4th day 1,007 mg/ml, *L. plantarum M* 0,915 mg/ml, on the 4th day 0,827 mg/ml, *L. plantarum T* 1,325 mg/ml, on the 4th day 1,235 mg/ml, *L. plantarum L* 0,785 mg/ml, on day 4 - 0,635 mg/ml, *L. plantarum J* formed 0,957 mg/ml, on day 4 – 0,833 mg/ml, *Bacillus*.sp. 1,285 mg/ml, on day 4 - 1,030 mg/ml. The highest activity was detected in the culture fluid of *L. plantarum 5* bacterial strain which produced 1.850 mg / ml on day 3 and 1.452 mg / ml IAA on day 4., These data resulted in the selection of this bacterial strain with high phytohormone activity for further studies (Figure 5).

As literature cites, GA ensures long-term preservation of green and fresh leaves, awakens seeds from dormancy, softens the endosperm layers, ensures the consumption

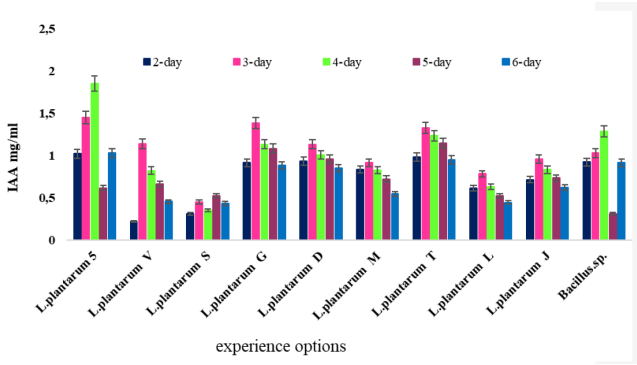

Figure 5: Quantitative analysis of IAA in the culture fluid of selected lactic acid bacteria (days 6).

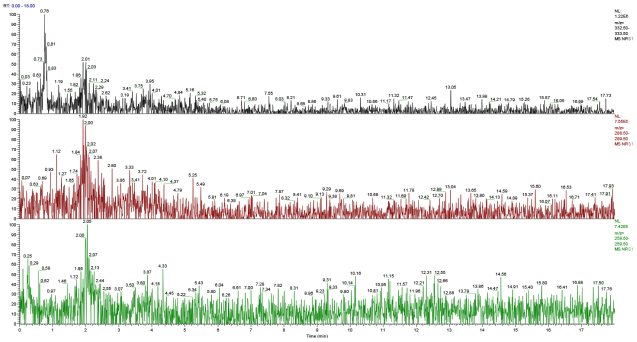

Figure 6: Mass spectrum of GA_4 gibberellin in *L. plantarum 5* bacterial strain culture fluid. Mass fragments detected in 2.00 min indicate that the substance is compatible with GA_4 gibberellin.

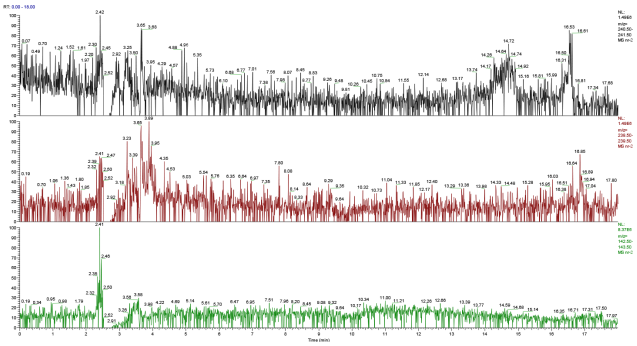

Figure 7: Mass spectrum of culture fluid of GA_7 gibberellin of *L.plantarum 5* bacterial strain. Mass fragments 2.41 min indicate that the substance is compatible with GA_7 gibberellin.

of reserves in it and enhances the development of the shoot[27] increasing the accumulation of nutrients and biomass in plants as well. Gibberellins are tetracyclic diterpenic acids in terms of their chemical structure, which are divided into groups containing 19 or 20 carbon atoms. The biosynthesis of gibberellins results in the formation of more than 100 substances, but not all of these substances have physiological activity.[28] GA_1, GA_3, GA_4 and GA_7 have been noted to be the most biochemically active gibberellins. Microorganisms synthesize growth-activating gibberellic acid and IAA, which enhances the development of the plant's root system.[20] During the study, a qualitative analysis of GA in the culture fluid of the local *L.plantarum 5* bacterial strain was performed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) method. The mass spectra of the chemicals in the extracted sums from the selected bacterial culture fluids were analyzed using a TSQ Quantum Access Max ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS) Ultimate 3000 Quaternary Standard (Thermo Fisher Scientific, USA) (Figure 6 and 7).

It is important to find the mass fragments of the substance under the investigation as a result of mass spectrometric analysis to determine GA_4 gibberellin. In the literature on mass spectrometric studies of this substance, we have tried to determine the presence of these ionic charges in our analyzes, using data on its formation of m/z 333, 289 and 259 ionic charges. The ionic charges released mass fragments detected in at 2.00 min in Figure 6 indicates that this substance is present in the extraction mixture as it complies with the literature data for GA_4 forming m/z 333, 289 and 259. Using this information, we concluded that GA_4 gibberellin was present in the culture fluid of the bacterial strain *L. plantarum* 5 studied in this work. Since the analyzes were performed directly using the culture fluids, the concentration of the substance appears to be very low. However, this was considered sufficient since the purpose of this analysis was to determine the presence of the formation of GA_4 gibberellin, but not its quantity, and we considered these data to be sufficient.

Analyses of *L. bactarum 5* bacterial strain culture fluid was found to contain GA_7 gibberellin as there were observed m/z 241, 239 and 143 mass fragments formed in 2.41 min (Figure 7), which also complies with literature data.[28]

CONCLUSION

There are a number of beneficial properties of microorganisms that are distributed in nature, the most important of which is phytohormone activity. GA plays a key role in plant development. The source of this phytohormone with growth properties are microorganisms. The role of lactic acid bacteria in industry is well known. In this study, the investigation of phytohormone activity of *Lactobacillus* bacteria isolated from different plants and their effect on plant development was carried out for the first time in Uzbekistan. Many of our scientists have studied the lactic

acid bacteria isolated from plants, but the effect of GA activity on plant development has not been thoroughly studied yet. According to the results of this study, the presence of GA_4 and GA_7, which stimulate plant growth and development, was detected in the culture fluid of *L. plantarum 5* bacterial strain using the chromato-mass spectrometrical method. In future, development of a biologically active preparation that enhances plant growth and an environmentally friendly product that is safe for the environment would be possible based on this research.

ACKNOWLEDGEMENT

Authors are grateful to the Institute of Genetics and Plants Experimental Biology, Academy of Sciences of Uzbekistan for providing space and resources to carry out this work.

Authors' contributions

All the authors substantially contributed to the conception, compilation of data, checking and approving the final version of the manuscript, and agree to be accountable for its contents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cite this article: Turaeva BI, Kamolova KHF, Soliev AB, Kutlieva GJ, Gibberellic and Indole Acetic Acids Producing Features of Bacteria from the Genus *Lactobacillus* and their Effect on Plant Development. Asian J Biol Life Sci. 2021;10(3):681-6.