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IAA-PRODUCING BACTERIA FROM THE RHIZOSPHERE OF CHICKPEA PLANT (*Cicer arietinum L.)* ISOLATION, CHARACTERIZATION, AND THEIR EFFECTS ON PLANT GROWTH PERFORMANCE

MSc THESIS

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JANUARY, 2024

WOLKITE, ETHIOPIA

Wolkite University
School of Graduate Studies

IAA-Producing Bacteria from the Rhizosphere of Chickpea Plant (*Cicer arietinum L.*) Isolation, Characterization, and their Effects on Plant Growth Performance

A Thesis Submitted to School of Graduate Studies, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant Biotechnology

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January, 2024
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APPROVAL SHEET

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We hereby certify that we have read and evaluated this Thesis entitled "**IAA-Producing Bacteria from the Rhizosphere of Chickpea Plant (*Cicer arietinum L)* Isolation, Characterization, and Their Effects on Plant Growth Performance"** prepared under our guidance by **Debebe Landina Lata.** Therefore, we recommend that the Thesis shall submitted as fulfilling the requirements for the award of Master of Science degree in Plant Biotechnology.

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This MSc thesis is dedicated to my beloved family, whose unwavering support and boundless encouragement have been the driving force behind my academic journey, serving as constant inspiration and sources of unwavering love.

DECLARATION

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ACKNOWLEDGEMENT

First, I would like to express my gratitude and sincere thanks to my honorable advisors, **Oumer Abdie** (Ph.D.), Department of Biotechnology, Wolkite University, and **Yayis Rezene** (Ph.D.), Southern Agricultural Research Institute (SARI), Hawassa, for their insightful advice and guidance from the proposal draft to the thesis completion. I would also like to express my special thanks and appreciation to my advisors for their guidance and valuable comments, sustained interest, stimulating views and encouragement during the writing up of this Thesis.

I am also very grateful to all Biotechnology department staff of Wolkite University for their positive collaboration of everything. I would like to convey my sincere gratitude, appreciation, and thanks to various institutions and individuals who assisted me and contributed to the successful completion of this study. I am highly indebted to my colleagues in different streams such as Biology (Mr. Samuel Adugna and Mr. Kaleb Kamayla), Food Processing Engineering Laboratory Assistances, and Chemistry (Mr. Ayansa Fikadu and Mr. Eyasu Milkias) from Wolkite University and Southern Agricultural Research Institute (Mr. Musa Tuke), Hawassa, in giving me different chemicals and materials in due times. I would like to express my sincere gratitude to my colleagues Mr. Techale Tilet and Mr. Habtamu Gobezi from Hawasa University, Mr. Wondewsen Mitiku my senior MSc student from Wolkite University, Mr. Duge Galtsa from Arbaminch Agricultural Research Center, and my friends Mr. Gezahagn Mulatu from America who supported me in financial during my work.

Last but not least, I want to express my gratitude to all of my families for their unwavering support during this time.

ABBREVIATIONS AND ACRONYMS

ACC deaminase	1- Aminocyclopropane-1-Carboxylic Acid
ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BTM	Bromothymol Blue
CRD	Completely Randomized Design
CSA	Central Statistical Authority
UN	United Nation
N_2	Dinitrogen
EIAR	Ethiopian Institute of Agricultural Research
EIB	Ethiopian Institute of Biodiversity
FAOSTAT	Food and Agriculture Organization of Statistics
FAO	Food and Agriculture Organization of the United Nation
IAA	Indole-3-Acetic Acid
ipdC	Indole-3-Pyruvic Acid
KB	King's B Medium
LSD	List Significance Difference
LB	Luria Broth media
m. a. s. l	Meter Above Sea Level
µg/ml	Microgram Per Milliliter
mm	Millimeter
Mo-Fe	Molybdenum Iron Cofactor
NA	Nutrient Agar
OD	Optical Density
PGPR	Plant Growth Promoting Rhizobacteria
PGRs	Plant Growth Regulators
PCR	Polymerase Chain Reaction
rpm	Revolution Per Minutes
SPSS	Statistical Package for Social Science
TSI	Triple Sugar Iron
UV	Ultraviolet
YEMA-BTM	Yeast Extract Mannitol Agar Bromothymol Blue
YEMA	Yeast Extract Mannitol Agar

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ABSTRACT

Indole-3-acetic acid (IAA), a crucial plant hormone, regulates diverse physiological processes. Plant growth-promoting rhizobacteria naturally present in soil can enhance plant growth by producing IAA. This study aimed to isolate and characterize IAA-producing bacteria from the chickpea (Cicer arietinum L.) *rhizosphere and evaluate their effects on plant growth. A total of 118 bacteria were isolated from 54 chickpea rhizosphere samples collected from Gurage Zone, Ethiopia. Purified isolates were designated as GAC; a Salkowski colorimetric was used for IAA production; and Bergey's systematic bacteriology manual was used for biochemical examination. The highest IAA-producing isolates were grown under various conditions and* in vitro *screened for their growth promotion traits. A PCR investigation was performed to determine the presence of IAA and nitrogen-fixing genes, and the isolates were evaluated for greenhouse conditions. Out of 118, 27 isolates produced IAA and eight isolates with the highest IAA-production (22.88-26.47 µg/ml) were selected. Morphological and biochemical identification classified six isolates as* Pseudomonas *and two as* Bacillus*. Optimal conditions for IAA-production were observed at 500 µg/ml tryptophan, 35 °C, and pH 7.0. A 48-hour incubation was ideal, except for GAC-34 and GAC-73, which required 72 hours. GAC-2 isolate achieved optimal IAA production with sucrose (45.28 µg/ml)* and *lowest by GAC-92 with fructose (7.72 µg/ml), and GAC-91 isolate produced the optimum IAA level with tryptone (9.62* µg/ml) and *lowest by GAC-34 with peptone (2.81 µg/ml). Selected isolates demonstrated nitrogen fixation by producing ammonia, changing the medium to a dark blue/yellow color. The GAC-118 isolate exhibited maximum phosphate solubilization (11.00 mm). GAC-118 isolate confirmed the presence of nifH, nifK, and ipdC genes. Greenhouse experiments revealed that these isolates significantly enhanced chickpea growth parameters (P≤0.05) compared to the control. Thus, uses of IAA-producing bacteria from chickpea rhizosphere could enhance Ethiopian crop productivity; further molecular identification and field studies needed.*

Key words*: Bio-inoculants, Chickpea, Indole-3-acetic acid, Molecular analysis, PGPR, Rhizosphere*

1. INTRODUCTION

1.1. Background of the Study

The increasing world population has led to a growing demand for food production, particularly in developing countries where a continuous need for sufficient food supply (Lwin *et al*., 2012; Messele Admassie *et al*., 2021). A recent report by UN forecasts a 9.8 billion global population by 2050 (Ying & Ping, 2022), emphasizing the urgent requirement for agricultural modernization, especially in developing countries. Ethiopia, with diverse agroecology, can employ varied agricultural methods and grow a range of crops (Jima Degaga *et al*. 2020). Among these crops, grain legumes (pulse crops), including faba beans, field peas, chickpeas, lentils, grass peas, fenugreek, lupines in cooler highlands, and haricot beans, soya beans, cowpeas, pigeon peas, mung beans in warmer lowlands, play a vital role as the predominant grain legumes in Ethiopian agriculture (Agbodjato *et al.*, 2015; Gupta *et al.*, 2015). Apart from contributing to protein production in cereal-based diets, these legumes supply essential nutrients to the soil and enrich the agricultural system by promoting plant growth (CSA, 2012a, 2022b; Erana Kebede, 2020).

The chickpea (*Cicer arietinum L.*), is a member of the *Leguminosae* family and a significant pulse crop appreciated for its high protein content (Gedefawu Wubie & Musa Adal, 2021). It is a staple food crop widely grown in the tropics, sub-tropics, and temperate and semi-arid regions of the world. It was globally ranked as the third most important pulse crop, with an average yield of 913 kg/ha and a total production of 10.9 million metric tons (Mohammed Zehara & Fassil Assefa, 2021). It is currently cultivated in the regions of Amhara, Oromia, the Southern Nations Nationalities and People's Region (SNNPR), and Tigray. Around 93% of Ethiopia's total chickpea crop is produced by the Amhara and Oromia regions combined, while 3.5% and 3%, respectively, are produced in SNNPR and Tigray (Menale Kassie *et al.,* 2009; Wondwosen Tena, 2017). It is grown alternately with main crops such as teff (*Erograstic Tef*), wheat *(Triticum sp.*), and barley (*Horduem vulgar*) in a traditional low-input agricultural system that suffers from soil nutrient deficiencies (Gemechu Keneni, 2012). This is due to its ability to biologically fix nitrogen and improve soil fertility *(*Alemayehu Getahun *et al*., 2020).

In order to fulfill food demands, farmers are currently using agrochemical goods such chemical fertilizers, herbicides, fungicides, and insecticides excessively to increase crop

yield (Ali *et al*., 2015; Zerihun Tsegaye *et al*., 2019). However, the application of agrochemical goods to achieve satisfactory results is now threatening agricultural processes, such as pollution of large water resources, destruction of microorganisms (rhizosphere biodiversity), reduction in soil fertility, and a risk to human health and the environment (Sadhana *et al.*, 2017). Regarding the damage caused by agrochemicals, scientists around the world are exploring new approaches. Among the explored approaches, the use of a microorganism called Plant Growth Promoting Rhizobacteria (PGPR) is in pole position (Agbodjato *et al.*, 2015).

The rhizosphere, the area of soil around plant roots, is a rich source of microbes that can be used to develop bio-inoculants to promote plant growth and yield (Ali *et al*., 2015). Bacteria are the most common microbes in the rhizosphere, and they use nutritional substances (vitamins, amino acids, and others) released from plant roots for growth (Reetha *et al.,* 2014). The interaction between plants and microbes can be beneficial, and bacteria isolated from the rhizosphere are known as PGPR (Reetha *et al*., 2014; Yousef, 2018). PGPR can directly or indirectly enhance plant growth by producing phytohormones (i.e., auxin), nitrogen fixation, phosphate solubilization, siderophores, hydrogen cyanide (HCN), hydrolytic enzymes, and antibiotics (Kejela *et al*., 2016). A diverse group of species belonging to the genera *Pseudomonas, Azotobacter, Acinetobacter, Agrobacterium, Aeromonas, Azospirillum, Klebsiella, Rhizobium, Enterobacter, Bacillus, Xanthomonas, Arthrobacter, and Serratia* were to be PGPR (Lebrazi *et al.*, 2020). Among these organisms, *Pseudomonas and Bacillus* species have been studied for their plant growth promotion (PGP) activity, mainly because of their production of IAA, siderophores, and antibiotics (Kaur & Sharma, 2013). The most common and well-studied plant hormone is IAA, and it is estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Ali *et al*., 2015).

Indole-3-acetic acid (IAA) is one of plants' most naturally occurring active auxins (Zerihun Tsegaye *et al*., 2019). Rhizospheric bacteria, in particular, are more likely to synthesize and release auxin as secondary metabolites due to the abundance of root-secreted substrates (Sadhana *et al.*, 2017). Microbes synthesize IAA by using both tryptophan-dependent and tryptophan-independent pathways, as established by researchers (Mohite, 2013; Damam *et al.*, 2016; Akter *et al*., 2021). L-tryptophan is a more efficient precursor for IAA biosynthesis (Kafrawi *et al*., 2014). PGPR synthesizes IAA using various amino acid

tryptophan-dependent pathways, such as the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetamide pathway (IAAId), the tryptophan side chain pathway, the tryptamine pathway, and the indole-3-acetonitrile pathway (Keswani *et al.*, 2020). The IPyA pathway is the most important pathway for bacteria to biosynthesis IAA, a plant hormone that helps plants grow (Moreno *et al*., 2015; Zhang *et al.*, 2021; Shah *et al*., 2022). The IpdC gene encodes the indole-3-pyruvate decarboxylase enzyme, which is responsible for converting IPyA to AAId and then to IAA (Zhang *et al*., 2021).

The production of IAA by rhizospheric bacteria can impact various stages of plant growth, including seed germination, root elongation, growth of root hairs, shoot and root length, and the overall dry weight of both shoots and roots (Harikrishnan *et al*., 2014; Ali *et al.,* 2015; Babiye Birhanu, 2022). The production of IAA in culture media, however, is influenced by various factors such as pH, incubation period, concentration of tryptophan, and the type of carbon and nitrogen sources utilized (Mohite, 2013;Chandra *et al*., 2018; Shoukry *et al*., 2018).

Recent advancement into PGPR underscores their vital role in organic farming, sustaining soil fertility, and promoting plant growth (Yonas Tassew & Samuel Sahile, 2017). Scientific interest in the potential of PGPR in agriculture has been steadily increasing, providing an attractive alternative to costly synthetic fertilizers and pesticides (Sadhana *et al.*, 2017). In resource-poor regions like Ethiopia, where inorganic fertilizers are challenging due to high costs, exploring alternatives becomes imperative. IAA-producing bacteria are emerging as promising biofertilizers, particularly in tropical regions, offering a sustainable solution to enhance nitrogen availability in the soil and increase productivity. The growing interest in replacing agrochemicals with bacterial inoculants signifies a growing interest in more sustainable agricultural practices (Yonas Tassew & Samuel Sahile, 2017). Consequently, the outlook toward organic farming and biofertilizers presents a promising avenue for sustainable development in both developed and developing nations like Ethiopia. Therefore, the aim of this study was to isolate IAA-producing bacteria from the rhizosphere of chickpea plants in the Gurage Zone sub-districts.

1.2. Statement of the Problem

Today, the increasing global population has heightened the demand for food production, particularly in developing nations where a consistent food supply is crucial (Lwin *et al*., 2012; Singh *et al.*, 2014; Messele Admassie *et al.*, 2021). In Ethiopia, many farmers utilize soils for crop cultivation that lack the necessary nutrients for optimal plant growth. However, the frequent application of agrochemicals has resulted in soil fertility loss, disruption of microbial diversity, and poses risks to both human health and the environment (Zerihun Tsegaye *et al*., 2019). Despite the crucial role of IAA as a plant hormone regulating various physiological processes, there is a significant knowledge gap regarding how IAA-producing bacteria precisely promote plant growth and function as effective biofertilizers, particularly in the context of Ethiopia. Limited laboratory or field studies have explored the potential of PGPR and bioinoculants in promoting plant growth. The scarcity of information on the diversity, prevalence, and characteristics of these bacteria hinders the development of sustainable agricultural practices, and the specific impact of IAA-producing bacteria on plant growth performance remains unclear, lacking well-defined mechanisms and outcomes of bacterial-plant interactions.

The study aims to fill this gap by implementing a systematic approach to isolate, characterize, and assess these bacteria, thereby contributing to the development of effective biofertilizers for sustainable agriculture. The challenges associated with the use of chemical fertilizers, which pose threats to soil health, environmental sustainability, and human health, underscore the urgency of exploring alternative approaches such as biofertilizers. By investigating tailored interactions between IAA-producing bacteria and plants, the research aims to provide valuable insights that can be applied to enhance microbial ecology, advance agricultural practices, and contribute to sustainable crop management.

1.3. Objectives of the Study

1.3.1. General objective

The general objective of this study was to study indole-3-acetic acid producing bacteria from the rhizosphere of chickpea plants (*Cicer arietinum L.*) and to evaluate their effects on chickpea plant growth performance.

1.3.2. Specific objectives

The specific objectives of the current work were:

- To isolate and characterize IAA-producing bacterial isolates from chickpea (*Cicer arietinum L.*) rhizosphere soil and root nodules under *in vitro* conditions;
- To optimize IAA production and to evaluate IAA-producing bacteria isolates for their plant growth promotion traits under *in vitro* conditions;
- To detect molecular analysis to determine the presence or absence of IAA-producing and nitrogen-fixing-related genes in bacteria isolates; and
- To evaluate the growth response of chickpea plants to inoculation of selected IAA-produced bacterial isolates under greenhouse conditions.

1.4. Significance of the Study

The isolation and characterization of IAA producing bacteria from the rhizosphere of chickpea plants represent a critical exploration with profound implications for agricultural practices. The significance of this study lies in its potential to unveil novel bacterial strains capable of synthesizing IAA, a key plant growth hormone. Understanding and characterizing these bacteria not only contribute to the expanding knowledge of microbial interactions in the rhizosphere but also present practical applications in sustainable agriculture. The evaluation of their effects on plant growth performance provides valuable insights into the potential use of these bacteria as biofertilizers or plant growth-promoting agents, which can enhance crop productivity while reducing the dependence on synthetic chemicals. This research addresses the growing need for eco-friendly and efficient approaches in agriculture, emphasizing the importance of harnessing the beneficial interactions between plants and rhizospheric microorganisms for improved crop yield and environmental sustainability.

2. LITERATURE REVIEW

2.1. Origin, Distribution and Production of Chickpea Plant (*Cicer arietinum L.*)

Chickpea (*Cicer arietinum L.)* belongs to the Leguminosae family and is an important legume valued as a rich source of protein (Mulissa Jida & Fassil Assefa, 2013; Gedefawu Wubie & Musa Adal, 2021). It most likely originated in present-day southeastern Turkey and adjacent Syria, where several of its natural species occur (Funga *et al*., 2016; Maesen & van der Jir, 2022). The crop later spread to India, Europe and subsequently reached Africa, Latin American and Central American countries. Chickpeas are a self-pollinating annual plant that can complete its life cycle in 90 to 150 days, depending on the prevailing meteorological conditions. Chickpeas can be grown under a variety of agro-climatic conditions around the world. Chickpea growing areas can be divided into the following four main geographical regions: Indian subcontinent; West Asia, North Africa and Southern Europe; Ethiopia and East Africa; and America and Australia (Funga *et al*., 2016).

Currently, chickpeas are produced in more than 60 countries worldwide and are the world's third most important grain legume after the common bean with particular importance in the semi-arid tropics of sub-Saharan Africa and South Asia (Zafar, 2017) estimated that the crop contributes to more than 20% of the world production of legumes, covering 15% of the total land area (Mohammed Zehara & Fassil Assefa, 2021). Ethiopia is considered to be the center of chickpea diversity (Gemechu Keneni, 2012) and is mainly grown in Central, North and South eastern highland areas of the country with an altitude of 1500-2700 m.a.s.l. with an annual rainfall of 500-2000 mm and an average temperature of 1025 °C. It prefers well-drained, sandy, sandy loam and black soils with a pH of 5.5-7.5. Under these conditions, the crop takes 90-150 days to mature. Chickpea seeds are high in protein and the seeds are eaten as green vegetables (green unripe seeds), kollo (soaked and roasted seeds), and nifro (cooked seeds). Chickpea sauces; Shiro (powdered seeds) and kik (split seeds) are commonly eaten with grain injera (pancakes). Bread could be baked from the ground seeds. This culture can also be used mixed with cereals and root crops in the preparation of baby food (Aynalem Birhanu *et al*., 2018).

In general, two types of chickpeas are produced worldwide: the Desi and the Kabuli types. Morphologically, the Desi type is characterized by small seeds with an angular appearance, sharp edges, pink flowers and different colors, but usually light brown and light. Although these two types of chickpeas are currently produced in Ethiopia, the Desi-type chickpea is traditionally and widely grown in the country. Kabuli-type production is restricted to a few pockets, mainly in the eastern Shewa Zone, and produces a higher yield under ideal conditions and is more sensitive to environmental pressures at lower yields (Admas Sintayehu *et al*., 2021). Recent research shows that chickpeas can fix more nitrogen than other legumes, improving soil fertility for subsequent crops. The rhizobacteria suitable for tuber formation on the roots of chickpeas differ from those that tuber other legumes. It is therefore crucial to inoculate chickpea seeds with the correct inoculant. Despite all these virtues and benefits, the productivity of this crop in Ethiopia's agriculture remains very low. There is also increasing evidence that more nitrogen can be fixed by existing legumes if they are inoculated more frequently or with more potent strains of rhizobacteria. Accordingly, if the full benefits are to be obtained from grain legumes in terms of maximum yield and soil improvement, the seed should be inoculated with its own specific and appropriate strain of rhizobacteria before planting (Muhammad *et al*., 2010).

2.2. Rhizosphere

The rhizosphere is the volume of soil under the influence of plant root excretions (sugars, organic acids and amino acids), which are very important and intensive interactions between microbes and plants take place. These interactions can significantly affect both plant growth and crop yields and have biotechnological applications (Esubalew Zegeye & Fassil Assefa, 2015). The microbiome of the rhizosphere is considered as a potential source of bacterial invasion into plant roots, which can provide different benefits to plants through different mechanisms, such as avoiding the harmful effects of phytopathogens (Koch *et al.,* 2010). These microbes are referred to as plant growth promoting rhizobacteria, which promote plant growth. As a result, PGPRs cover a wide range of bacterial taxa, including species from the genera *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Pantoea, Bacillus, Serratia*, and *Rhizobium* that have shown to be able to improve plant development (Esubalew Zegeye & Fassil Assefa, 2015).

In recent years, the use of PGPR has gained wide attention and acceptance worldwide due to its agricultural benefits (increasing crop yield) through the application of combinations of different modes of action, as they help to alleviate the dependence on the application of synthetic agrochemicals (Figueiredo *et al.,* 2016). Therefore, the rhizosphere is the most favorable area for the production of new agricultural inputs as bioinoculants or bio-promoters for crop production that have no harmful effects on the environment or human or animal health (Abawari, 2018).

2.3. Beneficial Role of Rhizosphere Microbes on Plants

Much resource has been devoted to research on PGPR to understand and exploit the microbial potential for plant performance. Understanding their mode of action will aid in the manufacture of microbial-based commercial growth-promoting products (Pratap *et al*., 2017). The beneficial role of microbes in the rhizosphere can be manifested by directly promoting plant growth, indirectly protecting against phytopathogens, and enhancing plant tolerance to certain abiotic stresses under suboptimal environmental conditions. The rhizosphere is a site of complex physical, chemical, and biochemical interactions, all of which affect plant growth (Gopalakrishnan *et al*., 2015). A brief list of the effects of this interaction includes: dinitrogen fixation, P mobilization and solubilization, nutrient uptake in deficient soils, improvement in water uptake, production of plant growth regulators, promotion of seed germination and early plant growth, improvement in soil structure, competition with plant pathogens, induced systemic resistance, general increase in biomass, and remediation of problematic soils (Saghir, 2012).

2.4. Plant Growth Promoting Rhizobacteria

The term PGPR is coined to refer to root-colonizing bacteria that cause growth and yield enhancement and to distinguish them from other mechanisms found in the rhizosphere that do not colonize roots or promote plant growth. Bacteria capable of promoting plant growth include those that live freely, those that form specific symbiotic relationships with plants (e.g., *Rhizobia* spp.), bacterial endophytes that form part or all of the inner tissue of a plant, and cyanobacteria (formerly called blue-green algae) (Thomas *et a*l., 2022). Bhattacharyya & Jha, (2012) reviewed that PGPR are the rhizosphere bacteria that promote plant growth

through a variety of mechanisms such as phytohormone production, phosphate solubility, siderophore production, biological nitrogen fixation, antifungal activity, induction of systemic resistance, promotion of beneficial symbioses of plant microbes, disruption of toxin production of pathogens, and others.

PGPRs are classified based on their functional activities as (i) biofertilizers (increasing nutrient availability to plants), (ii) phytostimulators (plant growth promotion, generally through phytohormones), (iii) phytoremediators (degrading organic pollutants) and (iv) biopesticides (control of diseases, mainly through the production of antibiotics and antifungal metabolites) (Gopalakrishnan *et al.,* 2015). Based on their relationship to host plants, plant growth-promoting rhizobacteria can be divided into two main groups as extracellular plant growth-promoting rhizobacteria (ePGPR) and intracellular plant growth-promoting rhizobacteria (iPGPR) (Gopalakrishnan *et al.,* 2015). The major ePGPRs could be found within the rhizosphere, at the rhizoplane, or in the spaces between cells of the root cortex, including *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Cauloba cter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas and Serratia* while another, iPGPRs found in root cells generally in specialized nodal structures including *Bacillus, Pseudomonas, Azotobacter, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium and Rhizobium* (Thomas *et al.*, 2022).

2.4.1. Characteristics of an ideal PGPR

A rhizobacteria strain is considered a putative PGPR if it possesses specific plant growth promoting properties and is capable of enhancing plant growth upon inoculation. The following requirements should be met by a perfect PGPR strain: (i) it ought to be highly environmentally friendly and rhizosphere-competent; (ii) upon inoculation, it should significantly invade the plant roots; (iii) it ought to be capable of encouraging plant development; (iv) it should be compatible with other bacteria in the rhizosphere; (v) it should be tolerant of physicochemical factors, and (vi) it should demonstrate better competitive skills over the existing rhizobacterial communities (Basu *et al.*, 2021).

2.4.2. Mechanisms of PGPR

As the dominant microbial community in the rhizosphere, PGPRs are actively or passively involved in promoting plant growth. PGPRs are divided into two categories according to their mode of action (Figure 1). The first are the so-called phytoprotective bacteria, which can protect the plant from pathogenic microorganisms through antibiotic synthesis and resistance induction (biocontrol) (Hooda & Rajni, 2013). The second groups are the phytobeneficial bacteria that improve plant growth through phytohormone synthesis (phytostimulation) and improvement of mineral supply (biofertilization). Some bacteria such as *Azospirillum, Pseudomonas, Xanhomonas and Rhizobium* including *Alcaligens feacalis, Enterobacter cloacae, Acetobacter diazotrophicus and Bradyrhizobium japonicum* can produce auxin (Rahman, 2018).

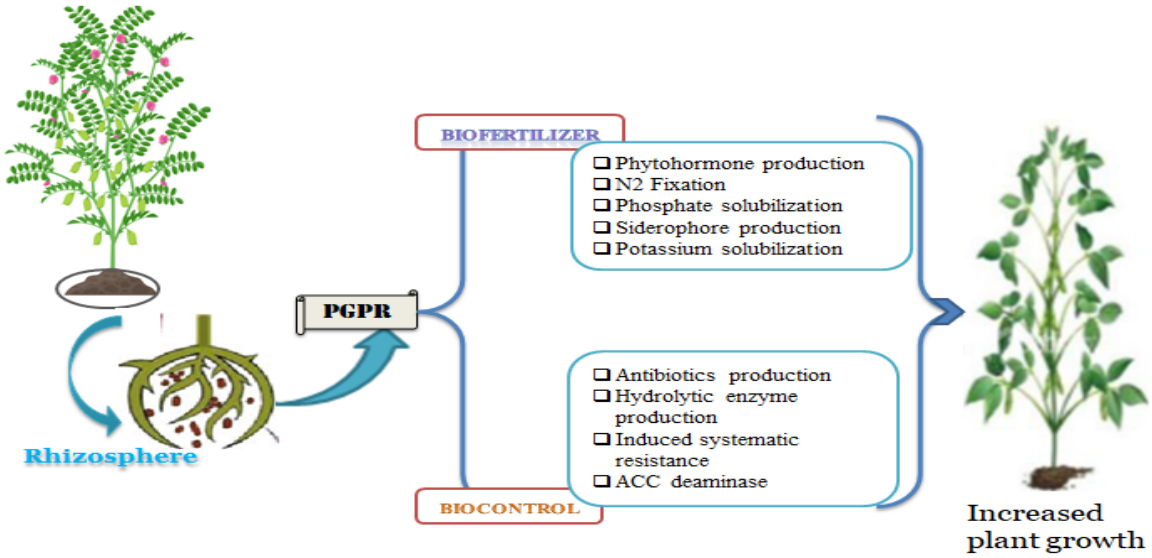

Figure 1.Promotion of plant growth by Rhizobacteria. Source: (Rahman, 2018).

The modes of action by which PGPRs promote plant growth have traditionally been classified into direct and indirect mechanisms, occurring inside and outside the plant, respectively. Direct modes of action of PGPR include improving plant nutrition by providing phytonutrients and development by regulating phytohormone levels (Basu *et al.*, 2021). The indirect effects of PGPRs include influencing the plant health through suppression of phytopathogens and other harmful microorganisms through parasitism, competition for nutrients and niches within the rhizosphere, production of antagonistic substances (such as hydrocyanic acid, siderophores, antibiotics, and antimicrobial metabolites) and lytic enzymes (such as chitinases, amylase, cellulose, glucanases, and proteases) (Ahmed Seid & Fassil Assefa, 2010).

2.5. Phytohormone Production by PGPR

Rhizobacteria, both symbiotic and non-symbiotic, can directly benefit plants by creating PGP activities that encourage plant development. Plant growth regulators, which control plant growth and development, are phytohomones that PGPR synthesizes and exports (Ali & Hayat, 2015). They are synthesized in one plant part and then transported to the other, where they may cause the physiological response and effect on growth and fruit ripening. A wide variety of microorganisms found in the rhizosphere can provide substances that direct plant development and advancement (Rahman, 2018). Phytohormones are signaling molecules that act as chemical messengers and play a fundamental role as growth and development regulators in plants. Auxins (i.e., IAA), cytokinin, gibberellins, and ethylene are the four main phytohormones produced by PGPR. More than 80% of bacteria in the soil and rhizosphere, including those with and without tryptophan, have the ability to generate auxin (IAA) (Esubalew Zegeye and Fassil Assefa, 2015).

2.5.1. Indole-3-Acetic Acid Production

IAA, a member of the phytohormone group, is widely recognized as the most important auxin in plants (Lwin *et al.*, 2012; Susilowati *et al*., 2018) and biosynthesized by fungi and bacteria. It is a common product of L-tryptophan metabolism in numerous microorganisms, comprising plant growth-promoting rhizobacteria. IAA regulates many aspects of plant development, including stem elongation, apical dominance, tropism, and lateral root initiation, as well as fruit, meristem, and root hair development through cell division and elongation (Mulissa Jida & Fassil Assefa, 2013; Aliciaa, 2014). Various mechanisms involved in growth enhancement by PGPR include microbial phytohormone production (IAA, cytokinin, gibberellins), enhanced water and nutrient uptake, enhanced soil nitrogen availability, phosphate solubilization, and siderophore production (Egorshina *et al*., 2012; Ahmed & Hasnain, 2014).

The rhizosphere bacteria capable of producing IAA contains of plant growth promoting bacteria such as *Azotobacter sp., Pseudomonas sp., Azospirillum sp., Rhizobium sp., Bacillus sp.* and *Enterobacter spp*. Biosynthesis of indole acidic caustic by rhizobacteria driving plant development involves assembly by means of indole-3-pyruvic acid caustic

and indole-3-acid aldehyde, which is the most widespread tool in microbes such as *Pseudomonas, Rhizobium, Bradyrhizobium, Agrobacterium, Enterobacter and Klebsiella*. The production of IAA by microbial isolates varies greatly between different species and strains and is dependent on substrate availability (Mulissa Jida & Fassil Assefa, 2013).

2.5.2. Mechanisms of Microbial IAA Production

The microbial synthesis of the phytohormone auxin has been known for a long time. The main precursor for the synthesis of IAA is tryptophan. The addition of tryptophan to culture media results in higher IAA production in all cases. Tryptophan biosynthesis begins at the chorismate metabolic node in a step-by-step process encoded by the trp gene. The branch point compound chorismate is synthesized from phosphoenolpyruvate and erythrose-4-phosphate in the shikimate pathway, a common pathway for the biosynthesis of aromatic amino acids and many secondary metabolites (Ramadan *et al.*, 2016).

Auxin IAA biosynthesis in bacteria can occur via multiple pathways, three of which are commonly observed, namely IAM (indole-3-acetamide), IAN (indole-3-acetonitrile), and IPyA (indole-3-pyruvic acid) pathways (Won *et al.*, 2011). IAA, synthesized by beneficial bacteria that promote plant growth, incorporates the IpdC gene that encodes indole-3-pyruvate decarboxylase, an enzyme that catalyzes the conversion of IPyA to indole-3-acetaldehyde. In the indole-3-acetamide pathway, the main pathway of IAA production in phytopathogenic strains, the synthesis of indole-3-acetamide from tryptophan is catalyzed by tryptophan-2-monooxygenase. Similarly, indole-3-acetamide hydrolase is the enzyme that catalyzes the conversion of indole-3-acetamide to IAA (Won *et al.*, 2011), while in plant growth-stimulating bacteria, the synthesis of IAA via the intermediate indole-3-pyruvic acid runs. In this pathway, tryptophan is converted to indole-3-pyruvic acid, which is decarboxylated to indole-3-acetaldehyde by indole-3-pyruvate decarboxylase, followed by IAA formation (Patten & Glick, 2002).

2.6. Biological Nitrogen Fixation

Nitrogen (N_2) is one of the most important macronutrients essential for the growth of all living organisms including plants and bacteria. Atmospheric N_2 is converted into plant-usable forms by biological N_2 fixation (BNF), which converts nitrogen into ammonia by

nitrogen-fixing microorganisms using a complex enzyme system known as nitrogenase (Thomas *et al.*, 2022). There are primarily two types of nitrogen-fixing microorganisms: On the one hand, free-living (non-symbiotic) bacteria, such as those found in cyanobacteria (blue-green algae), *Anabaena* and *Nostoc* and genera such as *Azotobacter, Beijerinckia* and *Clostridium*, contain the ***nif*** gene cluster, which codes for the genes of the nitrogenase enzymes and other proteins (Olanrewaju *et al.*, 2017).

The process is controlled by various **nif** genes along with other structural genes involved in iron protein activation, electron donation, iron-molybdenum cofactor biosynthesis, and many other regulatory genes responsible for nitrogenase synthesis and activity are required (Wewalwela, 2014). The other is mutual (symbiotic) bacteria such as Rhizobium related to legumes and numerous species of *Azospirillum* related to cereal grasses. This shows that the property of molecular nitrogen fixation is widespread in different genera of organisms, which differ greatly in terms of metabolism, physiology, morphology, genetic makeup and regulation (Olanrewaju *et al.*, 2017).

In symbiotic nitrogen fixation, microorganisms and plants coexist peacefully. The Rhizobium bacterial group which attaches to the roots of legumes and creates nodules where atmospheric nitrogen is fixed in a form that is available to plants specializes in symbiotic partnerships (Gopalakrishnan *et al.,* 2015). Non symbiotic nitrogen fixation is a characteristic of many heterotrophic bacteria that fix a significant amount of nitrogen without direct interaction with other organisms. *Azatobactor, Clostridium, Klebsiella and Bacillus* species are examples of this type of nitrogen fixation (Vadakattu & Paterson, 2006). Inoculation of biological nitrogen-fixing, plant growth-promoting rhizobacteria on crops offers an integrated approach to disease management, growth-promoting activities, and nitrogen maintenance in agricultural soils (Pailan *et al*., 2015) (Figure 2).

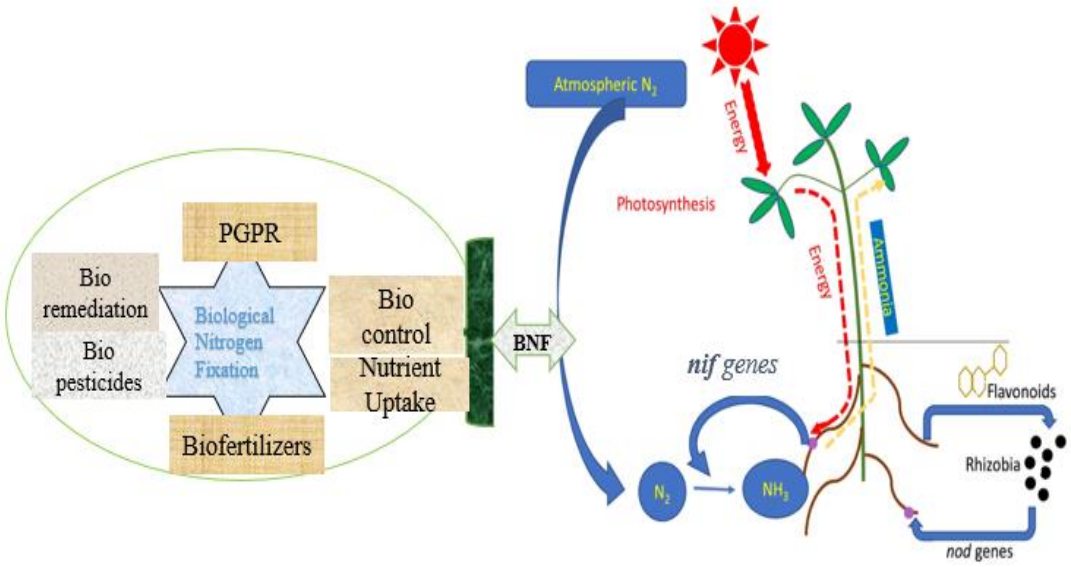

Figure 2. Model of nitrogen fixing bacteria showing the relationship among PGPRs: Source: (Pailan *et al.,* 2015).

2.7. Phosphate Solubilization

Phosphorus is the second most important macronutrient that plants need, after nitrogen, as it affects plant structure at the cellular level, stimulating both plant growth and productivity. Despite the large reservoir of inorganic and organic phosphates in most agricultural soils, about 95-99% of phosphorus is in insoluble, immobilized or precipitated form and is difficult to access for plants (Esubalew Zegeye and Fassil Assefa, 2015). Only a very low concentration of P is available to plants and it is generally deficient in most natural soils, being fixed as insoluble calcium phosphates in alkaline soils or as iron and aluminum phosphates in acidic soils. Plants absorb phosphate only in monobasic ($H_2PO_4^{-1}$) and dibasic (HPO_4^{-2}) form (Khandelval & Joshi, 2021).

To alleviate phosphorus shortages in soil, it is usually added to soils as chemical fertilizers synthesized through highly energy-intensive processes. Phosphate solubilizing bacteria convert soil inorganic phosphates such as $Ca_3(PO_4)_2$), $FePO_4$ and $AlPO_4$ and organic phosphates (inositol phosphate, phosphomonoester and phosphodiester) into a form easily accessible to plants by synthesizing low molecular weight organic acids, siderophores, hydroxyl ions and phosphatases. The production of organic acids, especially gluconic and carboxylic acids, is one of the well-studied mechanisms used by various phosphate-soluble soil bacteria (Sharma *et al*., 2013). Farmers have been interested in phosphorus solubilizing PGPB, which is found in the genera *Arthrobacter, Bacillus, Beijerinckia, Burkholderia,*

Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium, Rhodococcus, and *Serratia* (Rahman, 2018). Therefore, it is very beneficial to perform inoculation with phosphate-soluble bacteria that have multiple biosynthetic activities, or in mixed inoculation with other rhizosphere microbes with multiple plant growth-promoting properties (Gupta *et al*., 2015).

2.8. PGPR under Abiotic Stress Conditions

Changes in the environmental status of the rhizosphere can affect the survival and activities of PGPR. To this end, physiological approaches are an essential step to study PGPR activities and species identification. Low or high temperature is the most serious abiotic stress that limits nodule formation, nitrogen fixation, and plant growth and development. At temperatures below 10°C or above 37°C, most rhizobia grow in a limited area. Although the response to temperature is strain dependent, rhizobia are found to tolerate temperatures between 4 and 42.4°C (Hungria & Kaschuk, 2014). In vitro seed and soil treatment with *Pseudomonas fluorescens, P. fluorescens, Bacillus megaterium* and *Paenibacillus macerans* are said to reduce Fusarium wilt in chickpeas (*Cicer arietinum*) caused by *Fusarium oxysporum f. sp. ciceris* appeared at optimal temperatures for bacterial growth and production of inhibitory metabolites (Benaissa, 2019).

Soil acidity (pH) is a significant problem for agricultural production in many areas of the world and limits legume productivity. Acidic soil conditions pose problems for the plant, the bacteria and the symbiosis. Most legume plants require neutral or slightly acidic soil for their growth, particularly when dependent on symbiotic nitrogen-fixation-dependent environments. The highest level of nodule formation occurs in pH ranges between 5.5 and 7.5, while it is reduced below pH 5.5, with the exception of some acid-tolerant rhizobia strains (Benaissa, 2019). Therefore, selection of acid-tolerant rhizobia for inoculation of legume hosts under acidic conditions will ensure establishment of the symbiosis and also successful legume performance under acidic soils.

Energy in the form of ATP is produced through the carbon cycle. Photosynthesis is a crucial component of the nodulation process (Ahmed Seid & Fassil Assefa, 2010). Up to 25–40% of photosynthate produced by leguminous plants is released as root exudates. A portion of

the carbon is consumed directly to keep the nodules functioning, while a portion is transferred back to the host plant as carbon skeleton. The proper operation of nodules is hampered if the bacteroid lack their carbon source (Getahun Negash, 2015).

2.9. Molecular Analysis

Molecular analysis plays a crucial role in the identification and characterization of organisms, as it involves the examination of their DNA, RNA, and protein. By focusing on genes that exhibit high conservation within a species, scientists can effectively determine the functions of potential IAA producing and N_2-fixing microorganisms (Wewalwela, 2014). IAA is the most important and prevalent natural auxin produced by bacteria, fungi, and plants. L-tryptophan is the main source of indole production; the enzyme tryptophanase, which is encoded by the gene tnaA and converts Trp to pyruvate, ammonia, and indole in a reversible reaction, is controlled by the genes aroP, iacE, tnaB, trpE, tnaC, trpL, acrEF, and mtr9 (Sethia *et al.*, 2015). PGPR synthesizes IAA through various pathways, including the indole-3-pyruvic acid pathway, the indole-3-acetamide pathway, the tryptophan side chain pathway, the tryptamine pathway, and the indole-3-acetonitrile pathway, all of which depend on the amino acid tryptophan (Keswani *et al.,* 2020). The IPyA pathway is the most important pathway for IAA biosynthesis in bacteria. It has been found in a wide range of bacteria, including *Bacillus sp., Enterobacter sp., Azospirillum sp., Rhizobium sp., Pseudomonas sp.,* and *Arthrobacter sp*. (Moreno *et al.*, 2015; Zhang *et al*., 2021; Shah *et al.*, 2022). The ipdC gene is essential for the synthesis of indole-3-pyruvate in the IPyA pathway. In this pathway, tryptophan is oxidatively deaminated to indole-3-pyruvate, which is then converted to indole-3-acetaldehyde by the action of indole-3-pyruvate decarboxylase (encoded by ipdC) and then to IAA (Xiaobo *et al.*, 2022).

Nitrogen-fixing microorganisms in natural environments are detected using direct targeting of nifH genes by PCR with nifH-specific primers (Gaby & Buckley, 2014). Biological nitrogen fixation is a fascinating process that occurs in a variety of organisms. It involves the activation of specific sets of genes, known as **nif** genes, which play a crucial role in producing the enzyme nitrogenase. Nitrogenase is made up of two important protein components: the dinitrogenase reductase subunit (encoded by nifH or Fe protein) and the heterotetrameric core (encoded by nifK and nifD or FeMo-cofactor) (Gaby & Buckley,

2014; Wewalwela, 2014). In addition to these key genes, there are also regulatory genes like nifL and nifA, as well as electron transport genes (nifJ and nifF), genes involved in the assembly of the essential Fe-Mo cofactor, and genes responsible for processing the Mo-Fe protein. Analyses of microbial gene sequences that serve as functional biomarkers are facilitated by the availability of annotated databases of aligned sequences (Gaby & Buckley, 2014). Universal and group-specific PCR primers are used in quantitative real-time PCR for the quantification of gene copy numbers in the environment, for expression studies or for tracking organisms. Hence, many contemporary techniques in molecular microbial ecology require PCR and specific primers, which must be designed and evaluated using annotated sequence databases (Alviar *et al.,* 2021).

3. MATERIALS AND METHODS

3.1. Study Sites and Time

The experiment was conducted in the Molecular Biotechnology Laboratory, Department of Biotechnology, Wolkite University, Gurage Zone, and Southern Agricultural Research Institute (SARI), Molecular Biotechnology Laboratory, Hawassa, Ethiopia. The study sites of this research comprise four woredas of the Gurage Zone, including Abeshige, Kebena, South Sodo, and Sodo (Table 2) (Figure 3), which were selected based on the major chickpea production with no chickpea rhizosphere bacteria inoculation before. The rhizosphere samples (pink-colored nodules and soil from the roots and rhizosphere of a healthy standing plant, respectively) were collected between December 2022 and January 2023 during the late flowering stage.

Welkite (also transliterated Wolkite), is the capital town and separate woreda in South-western Ethiopia. The administrative center of Gurage zone, this town has latitude and longitude of (8°17′N 37°47′E) and about 158 km and 256.4 km far from Addis Ababa and Hawassa, respectively. Gurage zone comprises altitude ranging from 1001 to 3500 meters above sea level. Based on the local agro-climatic classification, the zone is classified in to three agro climatic zone. Dega (high altitude) covers 28.3% of the area and range between 2500-3662 mass, Weinadega (mid-altitude) at 1500-2500 mass encompasses about 64.9% of the area and kola (lowland) at 100–1500 mass covers 6.8% of the area. The average annual minimum and maximum temperatures and rainfall ranged from 18°C to 39°C and 450 to 820mm, respectively (“Welkite,” 2023).

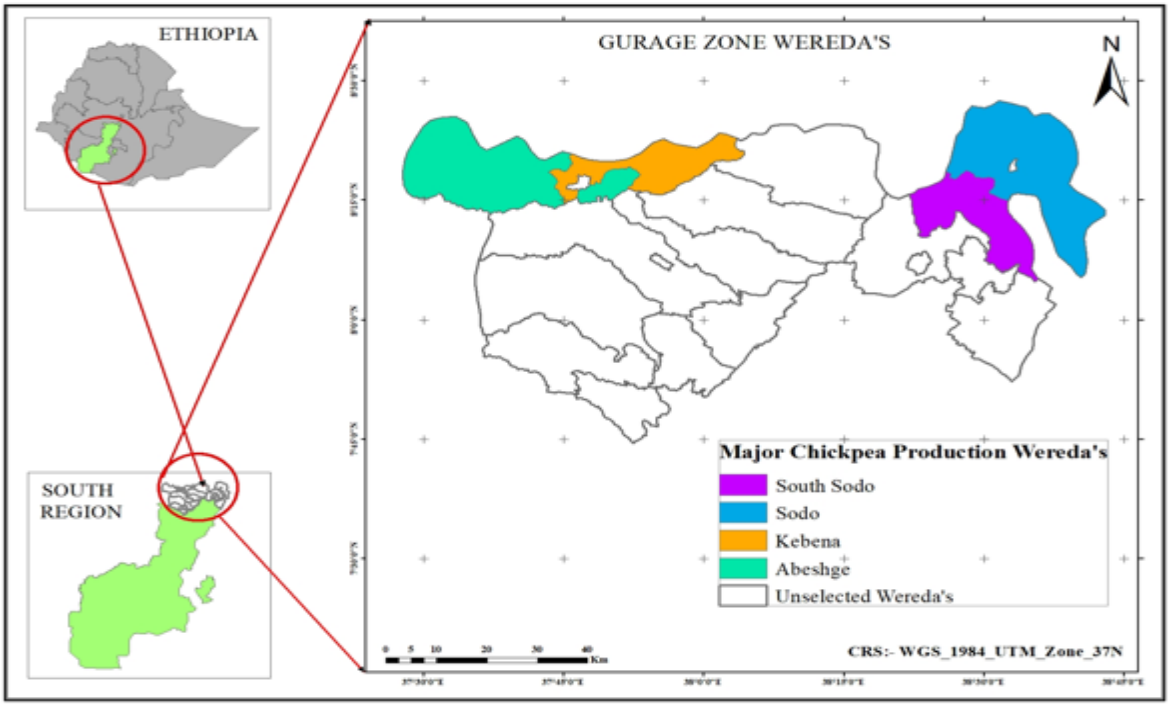

Figure 3. Map of chickpea nodules and soil samples collection sites/areas

3.2. Sample Size and Type

A total of 54 samples (24 root nodules and 30 rhizosphere soil) of cultivated chickpea varieties with potential producing composition were collected during the flowering stage from four selected Gurage Zone woreda (Table 1).

Table 1. Sample size and type

№	Sample type	Abeshige	Kebena	South Sodo	Sodo	Total samples
1.	The roots nodules of cultivated chickpea crops	7	6	6	5	24
2.	Chickpea rhizosphere soil	11	8	6	5	30
	Total	18	14	12	10	54

Table 2. Sampling sites for the collection of soil and root nodule samples of chickpea from selected woredas in the Gurage Zone of Ethiopia.

Number of isolates and Designation	Bacteria isolated samples	Sampling sites			Altitude (m. a. s l)	Location		Soil pH
		Adm. Zone	Woreda	Kebele		Latitude	Longitude	
2(GAC115-116)	Soil	Gurage Zone	Abeshige	Abuko	1663	8°20'36"N	37°40'70"E	5.77
2(GAC117-118)	Root nodule							
4(GAC21-24)	Soil			Darge	1533	8°24'57"N	37°73'17"E	5.63
1(GAC29)	Soil			L/Geraba	1882	8°16'31"N	37°48'13"E	6.02
4(GAC-25-28)	Root nodule							
4(GAC32-35)	Soil			Fenta	1771	8°16'24"N	37°42'32"E	7.06
2(GAC30-31)	Root nodule							
3(GAC7-9)	Soil			Jejeba	1825	8°15'41"N	37°44'11"E	7.21
3(GAC2-4)	Soil			Lache	1707	8°18'42"N	37°41'44"E	7.01
1(GAC1)	Root nodule							
2(GAC11-12)	Soil			Fite	1567	8°19'13"N	37°36'06"E	6.31
1(GAC10)	Root nodule							
2(GAC5-6)	Soil			T/Geraba	1815	8°15'48"N	37°47'15"E	6.43
1(GAC67)	Soil			Hudada 4	1565	8°21'59"N	37°31'28"E	6.57
2(GAC65-66)	Root nodule							
2(GAC36-37)	Soil			Michele	1749	8°18'53"N	37°44'20"E	5.64
3(GAC39-41)	Soil			Borer	1538	8°18'38"N	37°33'10"E	5.89
1(GAC38)	Root nodule							
3(GAC68-70)	Soil		Kebena	K/Kabada	1678	9°12'12"N	36°34'50"E	6.30
1(GAC71)	Root nodule							
2(GAC63-64)	Soil			Remuga	1830	9°15'10"N	36°28'44"E	6.20
4(GAC59-62)	Root nodule							
2(GAC44-45)	Soil			Fikadu	1855	9°18'61"N	36°54'24"E	6.38
2(GAC42-43)	Root nodule							
3(GAC56-58)	Soil			Rekaboka	1819	9°16'67"N	36°44'54"E	6.23
1(GAC55)	Root nodule							
2(GAC108-109)	Soil			Ferejete	1730	9°21'29"N	36°66'33"E	6.53
1(GAC108)	Root nodule							
4(GAC49-52)	Soil			Lencca	1884	9°19'75"N	37°10'79"E	6.17
2(GAC53-54)	Root nodule							
3(GAC46-48)	Soil			Wosharbe	1907	9°19'54"N	38°74'87"E	6.06
3(GAC104-106)	Soil			Zebimola	1867	9°17'28"N	36°84'81"E	6.32
4(GAC74-77)	Soil		Sodo	Negesa	2029	9°19'70"N	38°55'24"E	5.81
2(GAC72-73)	Root nodule							
1(GAC79)	Soil			Genbela	1927	9°12'63"N	38°50'74"E	5.93
1(GAC78)	Root nodule							
3(GAC80-82)	Soil			Anati	2045	8°19'79"N	38°56'29"E	5.86
1(GAC83)	Root nodule							
1(GAC89)	Soil			D/H/Gebeya	1839	8°98'32"N	38°53'56"E	5.25
1(GAC88)	Root nodule							
3(GAC84-86)	Soil			Firshi	2003	8°33'73"N	38°53'05"E	5.7
1(GAC87)	Root nodule							
2(GAC93-94)	Soil		South Sodo	Goget 1	1803	8°28'56"N	38°23'98"E	6.14
3(GAC90-92)	Root nodule							
2(GAC95-96))	Soil			Goget 2	1823	8°32'79"N	38°11'78"E	6.01
3(GAC97-99)	Root nodule							
1(GAC13)	Soil			Golbe	1904	8°53'34"N	38°45'06"E	6.30
3(GAC11-16)	Root nodule							
3(GAC18-20)	Soil			Goget 3	1842	8°56'06"N	38°34'12"E	5.97
1(GAC18)	Root nodule							
2(GAC113-114)	Soil			Agemsenad	1900	8°20'89"N	38°67'42"E	6.13
3(GAC110-112)	Root nodule							
3(GAC100-102)	Soil			K/Nurena	1901	8°43'13"N	38°56'07"E	5.89
1(GAC103)	Root nodule							

GAC= Gurage Auxin Chickpea, N = north, E = east, m. a. s. l = meter above sea level

3.3. Experimental Design of the Study

The study design was a completely randomized design (CRD) based experimental procedure with three replications for greenhouse to evaluate the efficiency of IAA-produced bacteria on the growth of chickpea seedlings.

3.4. Sample Collection

From selected woredas, the kebeles (smaller administrative districts within the woreda) with the greatest potential for chickpea farmlands were identified during the field investigation (Appendix 12). In these areas, chickpea have been growing for a long time without any history of inoculation with rhizosphere bacteria. Then, within each selected kebele, the farmers farmland with potential coverage with chickpea plant was selected and samples were collected triplicates randomly from the selected farmlands for the rhizosphere samples including both rhizosphere soil and root nodules (Appendix 1).

At each sampling site, the late flowering and early pod setting stages root nodule samples were detached from roots (cut the root 0.5 cm on each side of the nodule), and triplicates of chickpea root nodule were immediately put into sealed vials containing silica gel covered with 1cm of cotton wool from young and healthy chickpea seedlings for isolation of rhizosphere bacteria (Mulissa Jida & Fassil Assefa, 2013). For the rhizosphere soil sample, approximately 50g of soil was collected at a depth of 0-15cm from each sampling site using a sterilized spatula and transferred to an ethanol-sanitized (70%) polyethylene plastic bag. This depth was chosen because the P, N, and micronutrients found in such samples were directly related to nutrient uptake and plant growth (Zerihun Tsegaye *et al*., 2019; Mohammed Zehara & Fassil Assefa, 2021). Next, the complete set of chickpea root nodules and rhizosphere soil was transported right away to Wolkite University's Department of Biotechnology's Molecular Biotechnology Laboratory, where it was kept isolated at 4°C.

Chickpea seed accession 41209 was provided by the Ethiopian Institute of Biodiversity (EBI) for evaluating the effects of selected bacterial isolates on chickpea growth performance under greenhouse conditions (Table 3). The accession was collected from Amhara region, Siyadebrin Wayu Ensu woreda, which was characterized with local widely cultivated crop varieties and adaptable to the edaphic and climatic conditions. The chickpea

seedlings were prepared in the greenhouse experimental station at Wolkite University, Department of Biotechnology.

Table 3.Chickpea accession 41209 passport data

Accession. Number	Collection Number	Store Date	Region	Zone	Woreda	Kebele	Latitude	Longitude	Altitude	Crop	Genius	Species	Crop type
41207	RFG 91	2020-09-30	Amhara	Semen Shewa	Siyadebrin Wayu Ensu	Mitie	8°54'00"N	38°36'00"E	2580	Chickpea	*Cicer*	*Arietinum*	Pulse

3.5. Bacteria Isolation

For this study, rhizosphere bacteria was isolated from the chickpea (*Cicer arietinum L.*) rhizosphere by following the serial dilution plate method of (Sutrisno, 2021). One gram (1g) of soil was mixed with 9.0 mL of saline solution (0.85% NaCl) (w/v). The soil suspension was vortexed for five minutes to remove soil, stones, debris, and dead bacterial cells. Then, 1ml sample was serially diluted up to 10^{-7}. After that, 0.1 mL of each diluted sample was aseptically transferred to sterile Petri plates. The bacterial isolates were isolated on their respective media; *Pseudomonas* species on King's B agar and *Bacillus* species on nutrient agar medium. The plates were gently moved to mix the diluted soil solution uniformly with the medium and incubated for 48 hours at 28±2 °C.

In the process of isolating root nodule bacteria, dehydrated or desiccated root nodules were placed in labeled Petri dishes containing sterile distilled water overnight to absorb moisture. These nodules were then thoroughly washed with sterile distilled water to eliminate any surface contaminants. Next, the surface underwent sterilization using 70% ethanol for 10 seconds, followed by immersion in a 5% sodium hypochlorite (NaOCl) solution for 2-4 minutes. The nodules were subsequently rinsed 5-6 times with sterile distilled water to ensure complete removal of the surface sterilizing agents. The surface sterilized nodules were transferred into a sterile Petri dish one by one and crushed with an alcohol-flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood. A loop full of crushed nodule suspensions was streaked on a respective medium and incubated for 48 hours at 28±2°C (Appendix 2) (Ali *et al.*, 2015; Asnake Beshah & Fassil Assefa, 2019).

Single colonies were be picked from these plates and purified through repeated re-streaking on respective medium and incubated for 48 hours at 28±2 °C. All purified bacterial isolates were designated as GAC (Gurage Auxin Chickpea) with different numbers (i.e., 1, 2, 3 …118) representing each isolate. The isolates were preserved by two copies on slants medium; one copy was stored in 4°C refrigerators for the active work (short-term storage) and the other copy for long-term preservation in glycerol (50% v/v) at -20°C (Ali *et al.,* 2015).

3.6. Screening of Bacterial Isolates for IAA-Production

Production of IAA by bacterial isolates was used by Salkowski colorimetric assay according to the protocol proposed by Lebrazi *et al.*, (2020) with slight modification. Freshly grown cultures were inoculated into yeast extract mannitol broth (YEMB) media with three replications supplemented with 500 µg/ml L-tryptophan as the precursor of IAA. Bacterial cultures were incubated in a rotary shaker at 150 rpm at 28±2°C for 3 days. Approximately 1.5 mL of culture solution was then transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 10 min and 1 mL aliquot of the supernatant was mixed with 2-3 drops of orthophosphoric acid and 2 mL of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ into 49 ml of 35% perchloric acid) and the reaction tubes were incubated for 30 min in dark at room temperature. The development of a pink/pink-red color in the test tube indicated a positive result for IAA production. IAA concentration was determined from the supernatant absorbance using a spectrophotometer with a wavelength of 530 nm and calculated using IAA standard curve. The un-inoculated yeast extract mannitol broth media with Salkowski's solution was served control treatment.

3.7. Preparation of Standard Curve of IAA

In a test tube, different concentrations of IAA (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100µg/ml) were prepared in broth medium with three replications for each concentration. In a test tube, 1 ml of each IAA concentration was mixed with 2 ml of Salkowski's reagent (ratio 1:2) and incubated in the dark for 60 minutes at room temperature. After incubation, the solution turned pink, and the absorbance of the IAA standard solution was measured using a spectrophotometer at 530 nm. The standard curve of IAA concentration versus

absorbance was plotted and used to estimate unknown IAA concentrations (Sebaay & Ghomary, 2018).

3.8. Morphological and Biochemical Characterization of Bacterial Isolates

3.8.1. Morphological characterization of bacterial isolates

Bacterial isolates were examined for purity and colony morphology as well as culture characterization. According to the established techniques outlined in Bergey's Manual of Systematic Bacteriology (Bergey, 2008), the cell shape, size, color, and gram reaction were recorded.

Gram's Staining Reaction: A Gram stain reaction of the IAA-produced bacteria isolates was performed to verify whether the cultural purity of the test strain is gram-negative or gram-positive. A single colony of 48-hour-old bacteria isolates grown on respective agar plates was taken on a surface-sterilized microscopic slide to get smeared with one drop of water, gently smearing and following the standard Grams procedure. Then it was fixed by heating the slide on the spirit lamp. Then 2-3 drops of crystal violet solution were added to the smear and left for one minute. The slide was gently washed with distilled water, and 2-3 drops of iodine solution were added to the slide and incubated for one minute. The slide was washed with a few drops of 95% ethyl alcohol and counterstaining, 2-3 drops of safranin were added to the slide and left for 1 minute, and the slide was washed with distilled water and air dried. Finally, bacteria were studied under a microscope by using oil immersion (Bergey, 2008). Finally, bacteria colored purple was gram-positive, while bacteria colored red or colorless were gram-negative.

Motility Test: To conduct the motility test, a single, well-separated colony (18-24 hours old) was carefully picked using a sterile needle. The colony was then inserted into the middle of a tube, making a shallow stab of about one-third to half an inch. It was essential to maintain the same trajectory when removing the needle from the medium. The tube was incubated at a temperature of 37°C and observed periodically for 24 hours up to 7 days (Christopher & Bruno, 2015). A positive result was characterized by the presence of diffuse, hazy growth spreading away from the inoculation line, while a negative result showed growth confined to the inoculation line without any extension.

3.8.2. Biochemical characterization of bacterial isolates

All the isolates were tested for biochemical characterization (such as catalase, oxidase, gelatin liquefaction, indole, starch hydrolysis, citrate utilization, methyl red, Vogues Proskauer, urea hydrolysis, and the triple sugar iron test) (Sl *et al*., 2017).

Catalase Test: The test was carried out to study the presence of the catalase enzyme in bacterial colonies, as described by Reiner (2016). Freshly grown isolates were taken on a glass slide, and one drop of 30% H_2O_2 was added on a clean slide. The appearance of gas bubbles indicated the presence of the catalase enzyme, while the absence of bubbles indicated a negative reaction.

Oxidase Test: To perform this test, Kovac's oxidase reagent was used. Small pieces of filter paper were soaked in the 1% solution of Kovac 's oxidase reagent and dried, and a loopful of colony from a fresh 24-hour-grown isolate was taken and rubbed on the treated filter papers. The color was observed within 30 seconds. The development of a dark purple color indicated a positive result, while no color indicated a negative test (Al-joda & Jasim, 2021).

Starch Hydrolysis: Bacterial isolates were streaked on sterile starch agar plates as described by Su *et al*., (2018) and incubated at 28 ± 2°C for 24 - 48 h. After incubation, the plates were flooded with iodine solution, and the presence of zone hydrolysis on the plate indicated the ability of the test organism to metabolize starch.

Indole Production: The overnight culture of the isolates was inoculated on sterilized Sulfur, Indole, Motility (SIM) agar slants and incubated for 48 h at 28 ±2°C. Following incubation, 2 ml of Kovac's reagent was added to each tube. The isolates showing production of red color were positive for indole production, and yellow coloration indicates negative (Babu *et al.*, 2017).

Methyl Red Test: The test was done in Methyl Red-Voges-Proskauer (MR-VP) broth medium. The pure cultures of isolates were inoculated on broth medium and incubated at 37°C for 72 hours. After incubation, 3–4 drops of methyl red indicator were added and

gently shaken. Red color production was taken as positive, and yellow color production was taken as negative for the test (Mcdevitt, 2016).

Voges-Proskauer's Test: The test was done in Methyl Red-Voges-Proskauer (MR-VP) broth medium. The pure cultures of isolates were inoculated on broth medium and incubated at 37°C for 72 hours. After incubation, 3–4 drops of 40% KOH were added, followed by some 10 drops of 5% naphthol. The test tube was shaken and placed in a sloping position. The development of a red color starting from the liquid-air interface within 1 hour indicated a VP positive test, while no color change indicated a VP negative test (Mcdevitt, 2016).

Triple Sugar Iron Agar Test (TSI): Each isolate was inoculated by stabbing the medium with the aid of a sterilized straight wire loop, and the surface of the slope was inoculated by streaking and then incubated at 37 °C for 24 h. Gas production was determined by the cracking of the medium; formation of H_2S was determined by the blackening of the whole buffer or blackening at the slant butt junction; and glucose fermentation was determined by the yellowing of the butt. The fermentation of lactose and sucrose was determined by the yellowing of both the butt and the slant (Babu *et al*., 2017).

Citrate Utilization Test: Isolates were streaked on Simon's citrate slant agar and incubated at 37°C for 24 hours. The positive results for citrate utilization were indicated by the changing color of the media from green to an intense blue (Babu *et al*., 2017).

Gelatin Liquefaction: The overnight-grown isolates were inoculated into nutrient-gelatin deep tubes and incubated at 28°C for 24 h. Then the tubes were kept in the refrigerator for 30 minutes at 4°C. The isolates showing liquefied gelatin were taken as positive, and those that resulted in the solidification of gelatin on refrigeration were recorded as negative for the test (Edison & Martin, 2016).

Urea Hydrolysis: Isolates were inoculated on sterile urea agar slants and incubated at 37°C for 24 hours. Observations were made daily to distinguish positive and negative results. Positive results were confirmed by changing the color of the agar slant to pinkish

red and negative results remained no color change (yellowish color) (Sigurdarson *et al.,* 2018).

3.9. Optimization of IAA Production by Bacterial Isolates

To maximize the synthesis of IAA, bacteria isolates were grown under various conditions, including L-tryptophan concentration, incubation time, pH, temperature, carbon sources, and nitrogen sources (Chandra *et al*., 2018; Sebaay & Ghomary, 2018). The IAA production was quantified using the standard plot.

3.9.1. Effect of l-tryptophan concentration on IAA production

The effect of L-tryptophan concentration on IAA production by a selected bacterial isolate was studied on yeast extract mannitol broth media containing different concentrations of L-tryptophan (100, 200, 300, 400, 500, and 600 µg/ml) and incubated at 28±2°C for 3 days on a rotary shaker at 150 rpm (Lebrazi *et al*., 2020). After the incubation period, the cultures were centrifuged, and IAA production was estimated at 530 nm using the Salkowski reagent.

3.9.2. Effect of incubation period on IAA production

The effect of different incubation times (24, 48, 72, 96, and 120 hours) on IAA production by a selected bacterial isolate was studied on yeast extract mannitol broth media supplemented with L-tryptophan concentration (500 µg/ml) and incubated at 28±2°C on a rotary shaker incubator at 150 rpm (Bhutani *et al*., 2018). IAA production was estimated at 530 nm after every 24-hour interval using the Salkowski reagent.

3.9.3. Effect of pH value on IAA production

PH is one of the most crucial physicochemical factors in the synthesis of IAA. To achieve the ideal pH for IAA generation, the isolates were inoculated in a yeast extract mannitol broth media containing 500 µg/ml L-tryptophan at several pH values, such as 5, 6, 7, 8, and 9 (Shoukry *et al.*, 2018). IAA production was measured after 72 hours at 28±2°C and 150 rpm in a rotary shaker. IAA production was estimated at 530 nm using the Salkowski reagent.

3.9.4. Effect of temperature on IAA production

The development of bacteria is impacted by both low and high temperatures, and the proper growth of microorganisms is necessary for the generation of IAA. The effect of different incubation temperatures (25, 30, 35, 37, and 45°C) on IAA production by a selected bacterial isolate was studied on yeast extract mannitol broth media supplemented with L-tryptophan concentration (500 µg/ml) at 150 rpm for 72 h at 28±2°C (Bhutani *et al.,* 2018). After incubation, the supernatant was mixed with Salkowski reagent for color development, and IAA production was determined through the standard plot.

3.9.5. Effect of carbon sources on IAA production

Different carbon sources such as sucrose, fructose, glucose, and dextrose were added separately at 1% concentration to the yeast extract mannitol broth media containing 500 µg/ml L-tryptophan to obtain optimum IAA production by replacing mannitol (Sebaay & Ghomary, 2018). The culture was incubated in a rotary shaker at 150 rpm for 72 h at 28±2°C. After incubation, IAA production was estimated at 530 nm using the Salkowski reagent.

3.9.6. Effect of nitrogen sources on IAA production

Different organic nitrogen sources (peptone, tryptone, yeast extract, and beef extract) were added separately at 1% concentration to the yeast extract mannitol broth media containing 500 µg/ml L-tryptophan to obtain optimum IAA production by replacing yeast extract (Shoukry *et al.*, 2018). The culture was incubated in a rotary shaker at 120 rpm for 72 h at 28±2°C. After incubation, IAA production was estimated at 530 nm using the Salkowski reagent.

3.10. *In Vitro* Screening of IAA Producing Bacteria for Growth Production Traits

3.10.1. Phosphate solubilization activity

In the assessment of IAA-produced bacterial isolates, their phosphate solubilizing activity was screened in vitro using Pikovskaya's medium following the method outlined by Yadav *et al.*, (2014). The isolates were cultivated on specific media for 48 hours before being spot-inoculated on Pikovskaya (PVK) agar medium plates. These plates contained specific components per liter: glucose (10g), Ca3$(PO_4)_2$ (5g), $(NH_4)2SO_4$ (0.5g), NaCl (0.1g),

$MgSO_4.7H_2O$ (0.25g), yeast extract (0.5g), $MnSO_4.7H2O$, $FeSO_4.7H_2O$ (0.002g each), and 15g agar. After blending these constituents, the medium underwent autoclaving at 121°C for 15 minutes under pressure 106 Kpa, and the bacterial cultures were introduced onto the center of the agar plate using an inoculation loop under sterile conditions. These plates were then placed in an incubator for 2-3 days at 30°C, while plates without isolate streaks were employed as controls. A clear hallo zone surrounding bacterial growth indicated a positive outcome for phosphate solubilization.

3.10.2. Nitrogen fixation activity

The nitrogen-fixing activities of IAA-producing bacterial isolates were visually detected using glucose-nitrogen-free mineral medium (G-NFMM) with bromothymol blue (BTB) solution as a color indicator (Imran *et al*., 2021). On the G-NFM medium, pure bacterial colonies were streaked and cultured for 3 to 5 days at 30°C. After incubation, the ability of bacteria isolates to fix nitrogen was determined by the medium's color shifting from greenish blue to dark blue or yellow. When the generation of acid changes from green to blue or yellow, it means that the bacterial isolates are actively fixing nitrogen (Sulistiyani & Meliah, 2017). The composition of medium per liter includes: sucrose (20 g), K_2HPO4 (1 g), $MgSO_4$ (0.500 g), NaCl (0.500 g), Fe_2S0_4 (0.100 g), $Na_2MoO_42H_2O$ (0.005 g), $CaCO_3$ (2.00 g), Bacto agar (15.000 g), glucose (0.5%), and a trace amount of bromothymol blue as a pH indicator.

3.11. Molecular Analysis of IAA and Nitrogen-Fixing Related Genes

3.11.1. Genomic DNA extraction

Genomic DNA extraction was carried out by using a method of Andleeb *et al.*, (2022) by using phenol-chloroform with some slight modifications**.** The IAA-produced isolates (i.e., GAC-2, GAC-7, GAC-12, GAC-22, GAC-30, GAC-34, GAC-42, GAC-43, GAC-44, GAC-58, GAC-59, GAC-61, GAC-73, GAC-82, GAC-88, GAC-90, GAC-91, GAC-92, GAC-104, GAC-105, GAC-106, GAC-108, GAC-109, GAC-115, GAC-117, GAC-118, and GAC-24) were grown in LB broth and incubated on a rotary shaker at 120 rpm for 24 hours at 30°C. Each bacterial isolate was transferred to 1.5 mL Eppendorf tubes and centrifuged at 8,000 rpm for 5 minutes. The resulted pellet in each tube was resuspended and dispersed in a 250 µL Tris-EDTA (TE) buffer, 30 μL of 10% SDS and 3 μL of 20 mg/mL proteinase K. The mixers were mixed well by gently pipetting and incubate for 1hr

at 37°C. An equal volume of phenol: chloroform was added and mixed well by inverting the tube until the phases are completely mixed and centrifuged at 12,000 rpm for 10 min. The upper aqueous layer phase was transferred in to a new tube and $1/10^{th}$ volume of sodium acetate and 0.6 volume of isopropanol were added and mixed gently until the DNA precipitated. The mixers were spined at 8,000 rpm for 5 min. The DNA pellet was washed with 1ml of 70% ethanol and placed in freezer at 4°C for 15min and centrifuged at 8,000 rpm for 5 min. The supernatant was discarded and air-dry the DNA pellet. To be faster, it was drained well on to Kimwipe for 10 minutes and DNA resuspend in a 100 μL TE buffer. DNA was stored at 4°C short term and –20°C for long term (Alviar *et al.*, 2021). The concentration of each DNA sample was measured using spectrophotometric analysis. To evaluate DNA purity, the ratio of absorbance at 260 nm and 280 nm was employed. Typically, a ratio of ~1.8 to 2.0 is considered indicative of "pure" DNA. Deviation from this range, particularly a lower ratio in either case, may suggest the presence of substances like protein, phenol, or other contaminants that exhibit strong absorption near or at 280 nm.

3.11.2. PCR amplification of genes

PCR amplification of nitrogen-fixing and IAA-related genes was performed using the PCR primers: for the nifH gene, nifHF-5'-GGCAAGGGCGGTATCGGCAAGTC-3' and nifHR-5'-CCATCGTGATCGGGTCGGGATG-3' at 61°C (300 bp) (Sulistiyani & Meliah, 2017), the nifK gene, nifKF-5'-CCTGGATGACCGAAGACGC-3' and nifKR-5'-GGTGCCGCCTTCATACAT-3' at 59.95°C (360 bp) (Dai *et al*., 2014), and the ipdC gene, ipdCF-5'-AGAAGTCGCCGGTCGTCGTCAT-3' and ipdCR-5'-CCGCCAGTCGTCCAGGTCATTG-3' at 59.97°C (1170 bp) (Moreno *et al.*, 2015). The amplification conditions were optimized to achieve a high level of consistency in band patterns, utilizing agarose gel electrophoresis with a concentration of 1.3% (w/v). PCR amplification was carried out in 25µL volume, including 0.5µL of 10mmol/l dNTPs, 2.5µL of 10×buffer ($MgCl_2$+), 0.2µL of 5U/µL Taq polymerase, 1µL of 10µmol/L of each gene primer, and 2µL of 50ng/µL genomic DNA. Nuclease-free water was used to achieve 25µL of the total volume. The PCR conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation for 20 seconds at 94°C, annealing for 30 seconds depending on the annealing temperature of the gene primers, elongation for 1 minute at 72°C, a final extension for 10 minutes at 72°C, and a 4°C holding step for a maximum of 24 hours.

3.11.3. Agarose gel electrophoresis

The amplified DNA was separated by electrophoresis in 1.3% (w/v) agarose gel run in 1 × TAE buffer by using a Gel Doc UV trans-illuminator according to methods by Alviar *et al*., (2021) and Ratna & Meliah, (2017) with slight modification. A 1.3% concentration of agarose gel was prepared by adding 1.3 g of agarose powder to 100 ml of Tris-acetate-EDT buffer. By heating the mixture on a hot plate while stirring it with a magnetic stirrer, the mixture was thoroughly dissolved. 2 µL of ethidium bromide was added at around 50ºC and mixed by simple shaking. After that, the agarose was added to the horizontal electrophoresis casting apparatus. The 8 µL of DNA ladder (100 bp) was loaded into the first well of the agarose gel, and then the 6 µL of PCR products were properly mixed with 2 µL of 6X loading dye on parafilm before being loaded into the subsequent parallel wells. The horizontal gel electrophoresis was carried out at a constant voltage of 90 V for 90 minutes to 2 hours till DNA fragments were well migrated. The resulting amplicons were subjected to agarose gel electrophoresis to visualize and confirm the presence and absence of the genes at the expected molecular weight.

3.12. Greenhouse Evaluation of IAA Produced Isolates for Growth Promotion

3.12.1. Inoculum preparation

The eight highest IAA-produced bacterial isolates were selected for greenhouse evaluation in the Department of Biotechnology, Wolkite University. Flasks with a capacity of 250 ml were selected and filled with 150 ml of the respective broth media. They were sterilized using the steam sterilization process and cooled in the hood. Then, a single colony of pure bacteria isolates was grown in 25 ml of the respective medium at 28±2°C for 48 h in the shaking incubator by setting the speed to 150 rpm. After 48 hours of incubation, the standard concentration was adjusted at $1x10^9$.

3.12.2. Plant growth promotion in pot experiment

The effects of eight selected highest IAA-produced bacteria inoculation on plant growth in chickpea 41209 accession were evaluated in pot experiments. The experiment includes non-inoculated soil (control) and five seeds per pot treated with *Pseudomonas* and *Bacillus* isolates. Pots were kept under pot experiment conditions till the end of the experiment

(Yadav *et al*., 2014; Gedefaw Wubie & Musa Adal, 2021). Six isolates of highest IAA-produced *Pseudomonas* (GAC-22, GAC-34, GAC-61, GAC-91, GAC-92 and GAC118) and two isolates of *Bacillus* (GAC-2 and GAC-73) from different soil and nodule samples were used. Therefore, nine treatments were used for the pot experiment (Appendix 11). The inoculation treatments were set up in a completely randomized design with three replications. For the pot experiment, those selected highest IAA-produced isolates were used as inoculum for seed inoculation. Seeds of chickpeas 41209 accession were sterilized by washing with 95% ethanol (v/v) for 30 s, followed by soaking in 5% bleach (v/v) for 3 minutes, and rinsing with sterile distilled water five times to remove extra bleach (Chandra *et al.*, 2018). The surface sterilized seeds of chickpea were soaked in a broth culture of *Bacillus* and *Pseudomonas* amended with sucrose (0.2%) to facilitate the adherence of the bacteria to the seeds, and other seeds were soaked in distilled water (which served as the control) for 30 min and dried for 15 min.

Plastic pots with a capacity of 3kg were filled with 2 kg of sterilized soil (sterilized for 15 minutes) and planted with five seeds, with three replications for each treatment. The control treatment was maintained with non-bacterial isolates. To meet the IAA needs of the plant, 30 ml of each treatment with a standard concentration of $1x10^9$ was added to the pot as a soil drench in equal amounts seven days later, when first and second leaves appeared and emerged. The pots were observed regularly and watered with sterilized water from time to time to establish the rich water conditions necessary for cultivation (evening and morning). Forty five days after sowing, the plants were harvested and measured for various plant growth variables like plant shoot height, plant shoot fresh and dry weight, and root length and root fresh and dry weight (Babiye Birhanu, 2022).

Plant shoot height and root length data were recorded by measuring the height and length in millimeters with a ruler. Data on the fresh shoot and root weight of both plants in shoot height and root length were recorded by weight measurement with a sensitive electronic balance in the unit of milligrams. Shoot and root dry weight data were recorded by drying the sample in a dry heat oven at 65°C for 4 hours and measuring the weight in milligram units using a sensitive electronic balance. The formula used to determine the increased percentage (%) of IAA-produced bacterial isolates for all agronomic parameters, in comparison to the control, is as follows:

$$\text{Increased \%} = \frac{\text{Treatment value} - \text{Control value}}{\text{Control value}} \times 100$$

3.13. Statistical Analysis

The mean data was computed in Microsoft Excel Window 11. Agronomic parameters (i.e., plant shoot height, plant shoot fresh and dry weight, and root length, root fresh and dry weight) from a pot experiment underwent statistical analysis using One-Way ANOVA at 0.05 using SPSS version 27. Each treatment was replicated three times. Furthermore, the relationship between agronomic parameters was assessed through Pearson correlation coefficient analysis. Optimization parameters were schemed using Origin Pro 2021.

4. RESULTS

4.1. Isolation and Screening of Bacterial Isolates for IAA Production

A total of 118 bacterial isolates were obtained, and among them, 74 isolates were isolated from the soil rhizosphere and 44 isolates were from the root nodule sample. 50 (42.37%) isolates of *Bacillus* spp. and 68 (57.63%) isolates of *Pseudomonas* spp. were obtained using the appropriate media growth. Out of 118 isolates, only 27 exhibited a positive reaction by developing a pink or pink-red color when reacted with Salkowski's reagent, which indicates a positive result for IAA production based on qualitative determination (Figure 4).

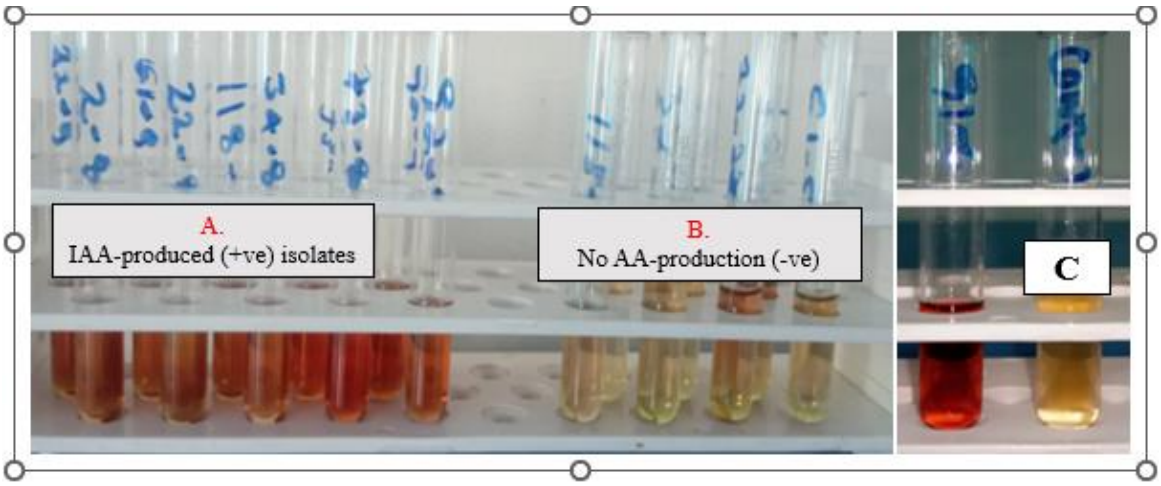

Figure 4. IAA production by bacterial isolates; (A): pink/red-pink color positive result; (B): yellow color negative result; and (C): red positive control and yellow negative control.

The IAA-produced isolates were screened for IAA production, which was determined by comparing them with the standard line curves of known IAA concentrations using Salkowski's reagent. A straight curve line (**y =0.0303x + 0.1185)** indicated a direct relationship between IAA concentrations and the extent of red color, as indicated in figure 4. The R^2 value of the graph was found to be 0.9704, which showed the validity of the graph (Figure 5) (Appendix 3).

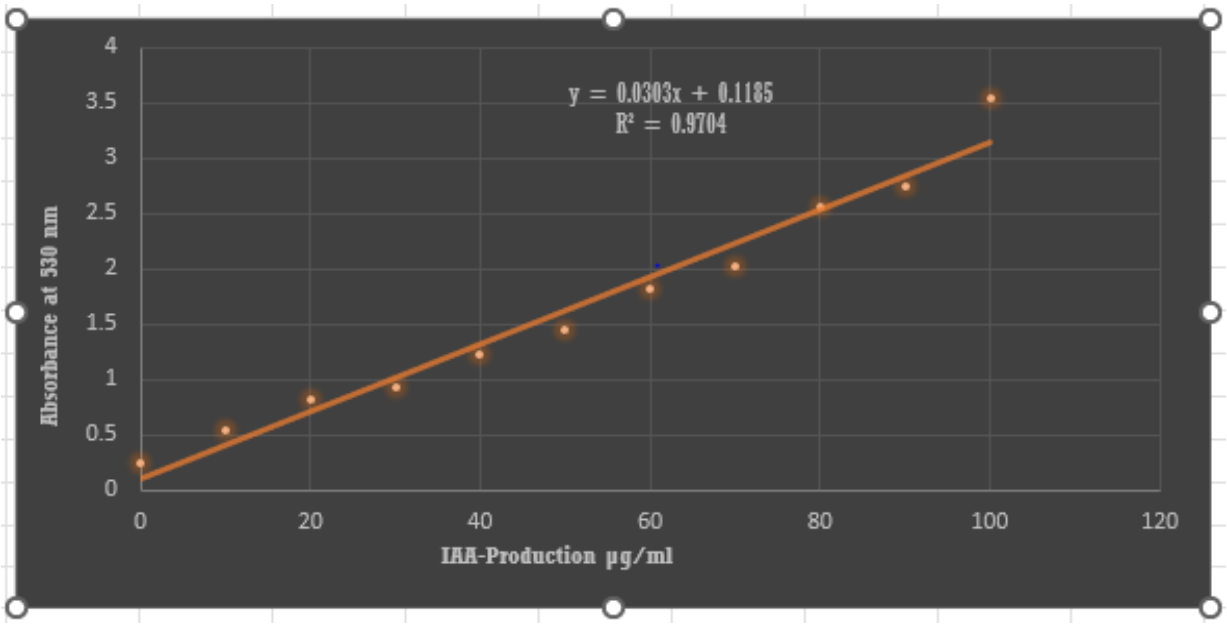

Figure 5. IAA Standard curve.

All 27 isolates produced IAA in the range of 2.15 µg/ml to 26.47 µg/ml (Table 4 and Figure 6). Among them, 8 isolates, GAC-2 (25.38 µg/ml), GAC-22 (24.83 µg/ml), GAC-34 (20.01 µg/ml), GAC-61 (23.95 µg/ml), GAC-73 (24.86 µg/ml), GAC-91 (24.95 µg/ml), GAC-92 (22.88 µg/ml), and GAC-118 (26.47 µg/ml) produced the highest concentration of IAA, and the lowest concentration was observed by GAC-117 (2.15 µg/ml) and GAC-105 (2.21 µg/ml). Those 8 isolates that produced the highest IAA were selected and identified depending on their morphological and biochemical characteristics, optimized with different culture medium parameters, and evaluated for greenhouse conditions. But all IAA-produced bacterial isolates were analyzed for the presence or absence of IAA and nitrogen-fixing-related genes.

Table 4.IAA production potential by bacterial isolates from chickpea rhizosphere samples

S. No	Isolates code	IAA Production (µg/ml)
1	GAC-2	**25.38±0.18**
2	GAC-7	4.49±1.14
3	GAC-12	8.36±0.99
4	GAC-22	**24.83±0.04**
5	GAC-30	5.49±1.11
6	GAC-34	**24.01±0.48**
7	GAC-42	3.52±0.69
8	GAC-43	8.14±0.08
9	GAC-44	7.28±0.58
10	GAC-58	7.06±1.18
11	GAC-59	11.43±0.07
12	GAC-61	**23.95±1.01**
13	GAC-73	**24.86±0.58**
14	GAC-82	4.87±1.15
15	GAC-88	5.85±0.06
16	GAC-90	11.84±0.58
17	GAC-91	**24.95±1.14**
18	GAC-92	**22.88±0.59**
19	GAC-104	11.80±0.58
20	GAC-105	2.21±0.60
21	GAC-106	5.58±0.14
22	GAC-108	11.91±0.62
23	GAC-109	11.88±0.78
24	GAC-115	12.42±0.50
25	GAC-117	2.15±0.18
26	GAC-118	**26.47±0.16**
27	GAC-24	3.93±0.67

IAA values are mean (n= 3) ± SD; GAC=Gurage Auxin Chickpea; IAA= Indole-3-Acetic Acid; SD=standard deviation

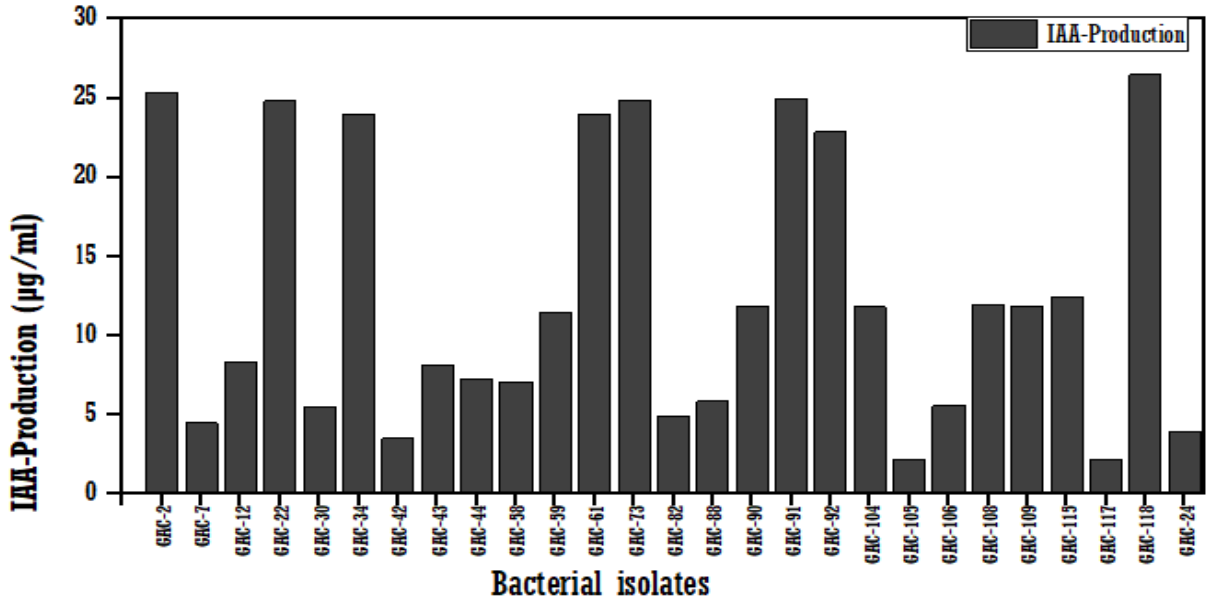

Figure 6. IAA production by bacteria isolates from chickpea rhizosphere samples

4.2. Identification of IAA Produced Bacterial Isolates

4.2.1. Morphological and cultural characterization

The morphological features of selected isolates on the nutrient agar plate were examined, and they revealed small to medium-sized and whitish-yellow gram-positive, motile, and rod-shaped cells. On the basis of biochemical reactions, these isolates (GAC-2 and GAC-73) were *Bacillus* species (Table 5) (Figure 7). The remaining six isolates were examined morphologically and named as *Pseudomonas* species. These isolates were showed features like small to medium, red, and yellowish white color with off-white (creamy white) around the colonies on King's B agar medium plate. Under microscopic examination, these isolates displayed characteristics such as small to medium sized, being gram-negative, being rod-shaped, and they were determined as motile. These isolates were named GAC-22, GAC-34, GAC-61, GAC-91, GAC-92, and GAC-118.

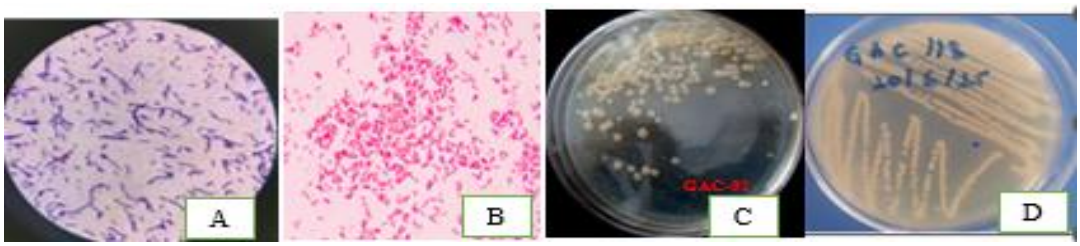

Figure 7. Morphological characterization of selected IAA-produced isolates: (A): gram-positive isolate; (B): gram-negative isolate; (C): GAC-92 isolate on KB's medium; and (D): GAC-118 pure isolate on petri plate.

Table 5.Morphological characterization of IAA-produced bacterial isolates

Isolates code	Morphological characterization				
	Colonial characterization			Cellular characterization	Motility
	Size	Shape	Color	Gram-staining (+/-)	
GAC-2	Small	Rod	Whitish yellow	+	Motile
GAC-22	Medium	Rod	Creamy White	-	Motile
GAC-34	Medium	Rod	Yellow	-	Motile
GAC-61	Medium	Rod	Yellowish white	-	Motile
GAC-73	Medium	Rod	Whitish yellow	+	Motile
GAC-91	Medium	Rod	Whitish yellow	-	Motile
GAC-92	Medium	Rod	Creamy White	-	Motile
GAC-118	Small	Rod	Yellow	-	Motile
+=Gram-positive bacteria; =Gram-negative bacteria					

4.2.2. Biochemical Characterization of Bacterial Isolates

All eight higher IAA-produced isolates were tested for biochemical characterization, oxidase, catalase, methyl red test, citrate test, starch hydrolysis, gelatin liquefaction, triple sugar iron test, urease test, Voges Proskauer's, and indole test (Table 6) (Figure 8). The two isolates (GAC-2 and GAC-73) were *Bacillus* species and both of them were commonly positive for catalase, methyl red, oxidase test, glucose, starch hydrolysis, and citrate tests, and negative for urease test, indole test, and lactose utilization. GAC-2 isolate was positive for gelatin liquefaction, sucrose, and VP test and negative for gas production and H_2S production. GAC-73 was positive for H_2S production and gas production and negative for gelatin hydrolysis, VP test, and sucrose utilization test. All six isolates (GAC-22, GAC-34, GAC-61, GAC-91, GAC-92 and GAC-118) of *Pseudomonas* species revealed positive results for catalase test, oxidase test, citrate test and glucose utilization and where as they were negative result for VP test, indole test, sucrose, and H_2S production. Methyl red test was positive for all *Pseudomonas* isolates except GAC-61. For gelatin hydrolysis all isolates were positive except GAC-91 and GAC-92. For urease test only two (GAC-91 and GAC-92) were positive result where the rest isolates were negative. For lactose and gas production test all the isolates were positive, except GAC-61 and GAC-92. For starch hydrolysis test three isolates (GAC-22, GAC-91, and GAC-92) showed positive results and remaining three isolates (GAC-22, GAC-34, and GAC-118) showed negative results.

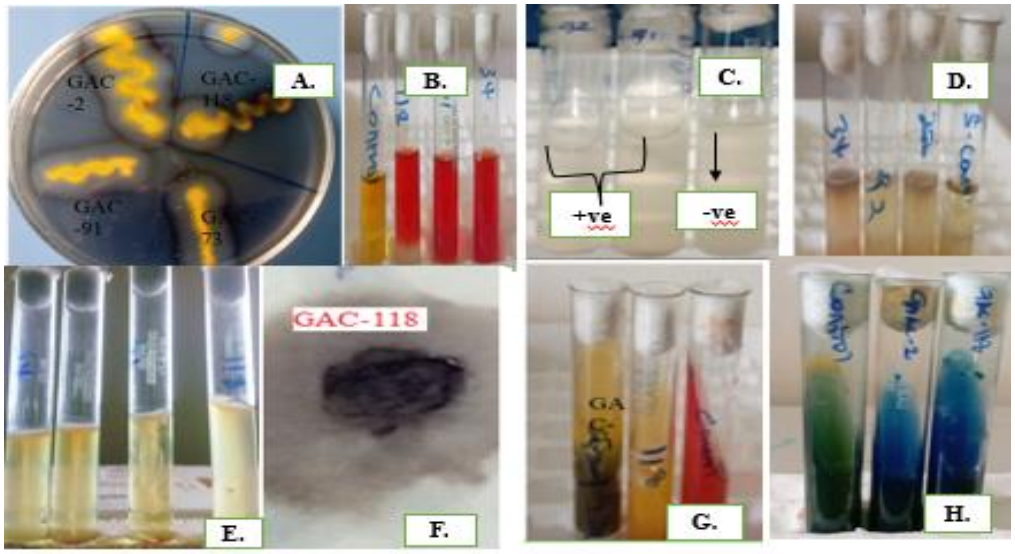

Figure 8. Biochemical characterization of selected IAA-produced isolates: (A): starch hydrolysis; (B): methyl red test; (C): gelatin hydrolysis; (D): Vogues-Proskauer's test; (E): motility test; (F): oxidase test; (G): triple sugar iron test; and (H): citrate utilization.

Table 6.Biochemical characterization of IAA-produced bacterial isolates

Biochemical characterization	**Isolates code**							
	GAC-2	**GAC-22**	**GAC-34**	**GAC-61**	**GAC-73**	**GAC-91**	**GAC-92**	**GAC-118**
Catalase	+	+	+	+	+	+	+	+
MR test	+	+	+	-	+	+	+	+
VP	+	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	-	-	+	+	+	-
Gelatin hydrolysis	+	+	+	+	-	-	-	+
Urease test	-	-	-	-	-	+	+	-
Oxidase	+	+	+	+	+	+	+	+
Gas production	-	+	+	-	+	+	-	+
H_2S production	-	-	-	-	+	-	-	-
Glucose	+	+	+	+	+	+	-	+
Lactose	-	+	+	-	-	+	-	+
Sucrose	+	-	-	-	-	-	-	-
Similarity of Bacteria species	*Bacillus* sp.	*Pseudomonas sp.*	*Pseudomonas* sp.	*Pseudomonas sp.*	*Bacillus* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.

GAC= Gurage Auxin Chickpea; VP=Vogues-Proskauer's; MR= Methyl red; H_2S=Hydrogen sulfide; += Production; - = No production

4.3. Optimization of IAA Production by Bacterial Isolates

4.3.1. Effect of tryptophan concentration on IAA production

Based on the spectrophotometric analysis, the results showed that increasing production of IAA along with L-tryptophan increased concentrations up to 500 µg/ml and then started to decrease production of IAA for all isolates (Figure 9) (Appendix 9). The maximum IAA production was observed on the media added with 500 µg/ml L-tryptophan for all isolates. The highest IAA was produced on isolate GAC-2 with 34.56 µg/ml at 500 µg/ml of L-tryptophan concentration, followed by isolate GAC-91 (33.88 µg/ml) and isolate GAC-118 (33.37 µg/ml). The lowest amount of IAA production was observed after 100 µg/ml (5.18 µg/ml) by the GAC-73 isolate.

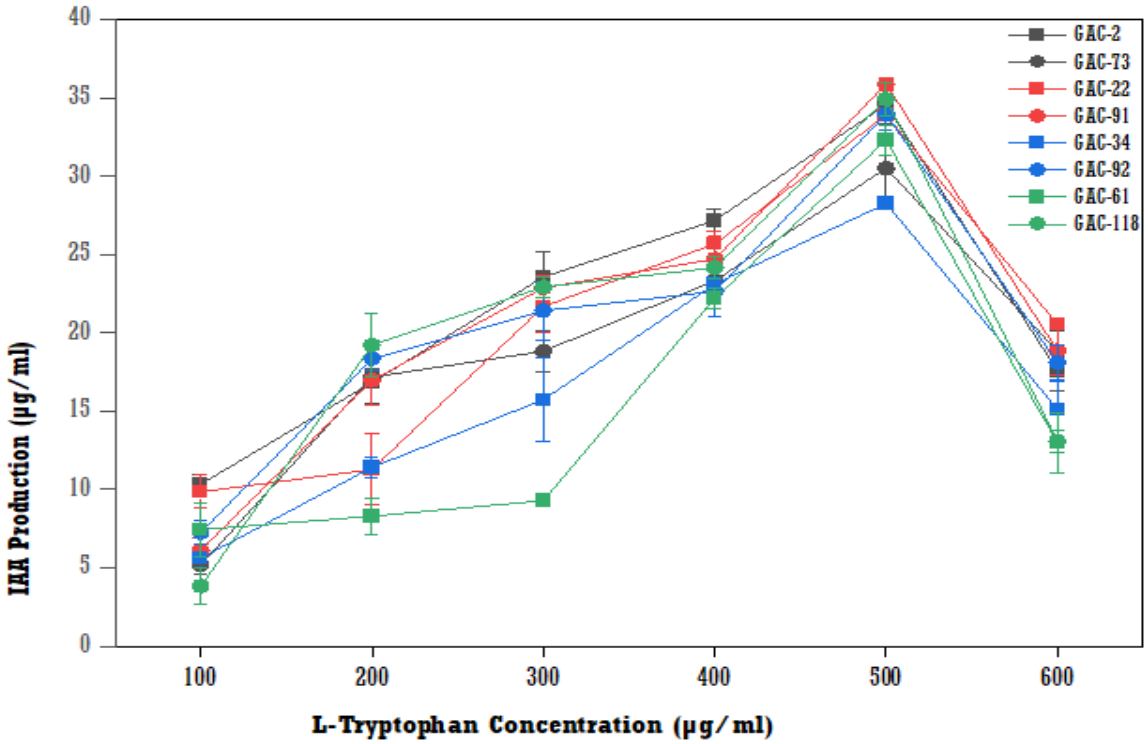

Figure 9.Effect of different L-tryptophan concentrations on IAA production by bacterial isolates (means (n = 3) ± standard deviation).

4.3.2. Effect of incubation period on IAA production

The production of IAA by isolates started 24 hours after the incubation period and reached a maximum IAA production after 48 hours for GAC-2, GAC-22, GAC-61, GAC-91, GAC-92, and GAC-118 isolates and 72 hours for GAC-34 and GAC-73 isolates, then the growth of bacterial isolates reached the stationary phase and decreased after 96 and 120 hours

(Figure 10) (Appendix 8). The maximum IAA production was observed after 72 hours of incubation (24.88 µg/ml) by the GAC-73 isolate, followed by GAC-22 after 48 hours of incubation (24.46 µg/ml). The minimum amount of IAA production was observed after 120 hours (3.55 µg/ml) by the GAC-22 isolate.

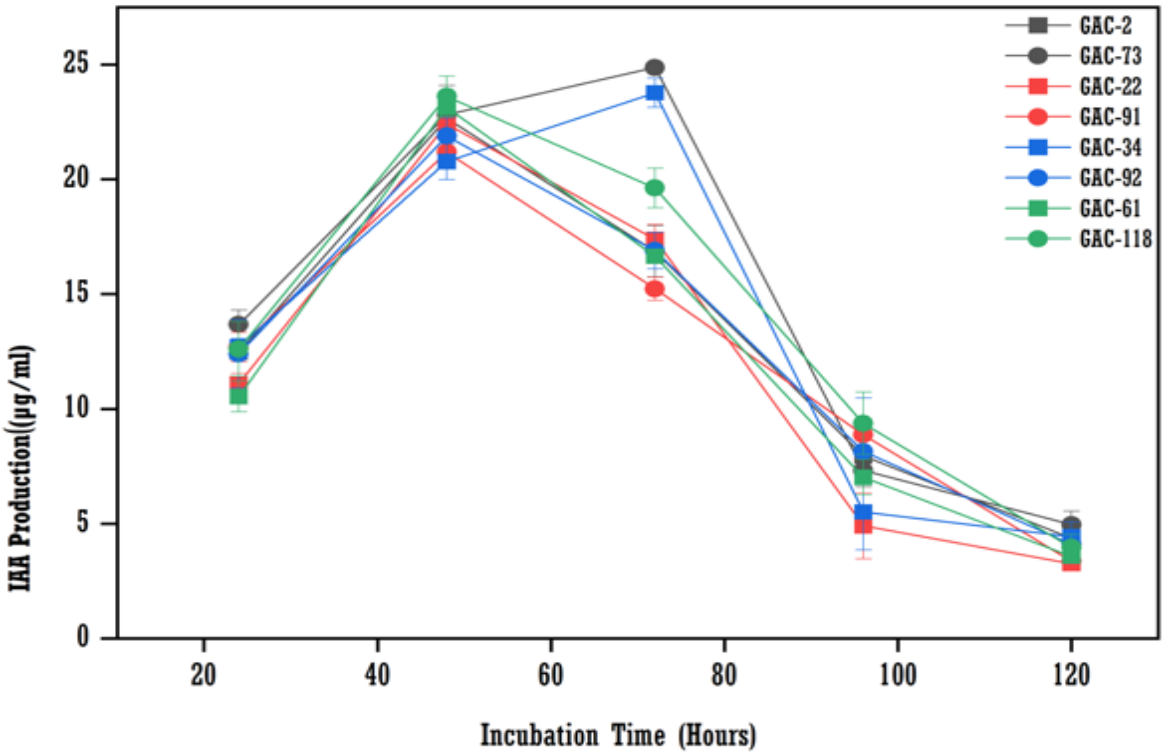

Figure 10.Effect of incubation periods on IAA production by bacterial isolates (means (n=3) ± standard deviation).

4.3.3. Effect on pH values on IAA production

IAA production results after incubation showed that pH 7 and pH 8 were the optimum pH for IAA production for all bacterial isolates (Figure 11) (Appendix 4). The maximum production of IAA was obtained on media with pH 7, followed by pH 8 and pH 6. The maximum IAA production was obtained at 61.85 µg/ml by GAC-91, followed by GAC-188 (60.62 µg/ml), and GAC-2 (60.56 µg/ml) at pH 7, and GAC-73 (51.48 µg/ml) and GAC-22 (46.04 µg/ml) isolates at pH 8, and the least IAA production was observed at pH 5 at 9.734 µg/ml by the GAC-34 isolate.

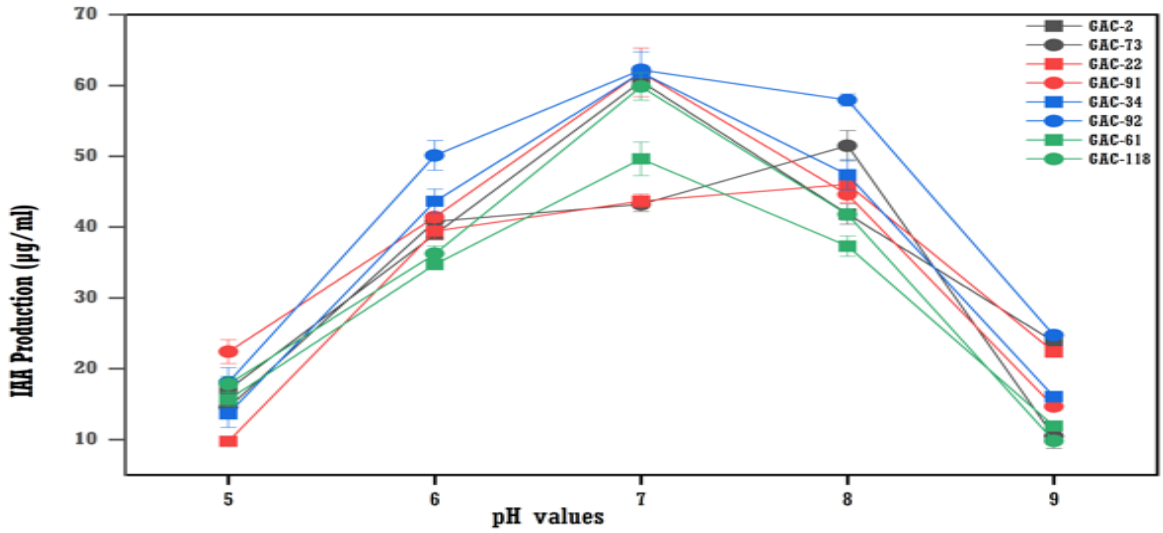

Figure 11.Effect of different pH values on IAA production by bacteria (means (n=3) ± standard deviation).

4.3.4. Effect on temperature on IAA production

The results obtained showed that the 35°C temperature was suitable for six bacterial isolates for maximum IAA production, except GAC-34 and GAC-61 at 37°C (Figure 12) (Appendix 5). The maximum production of IAA was yielded by the GAC-118 isolate with 52.89 µg/ml, followed by GAC-91 (45.89 µg/ml) and GAC-73 (44.32 µg/ml) at 35°C, and GAC-61 (35.66 µg/ml) and GAC-34 (34.94 µg/ml) at 37°C. The minimum IAA production was obtained by the GAC-92 (6.34 µg/ml) isolate at 45°C.

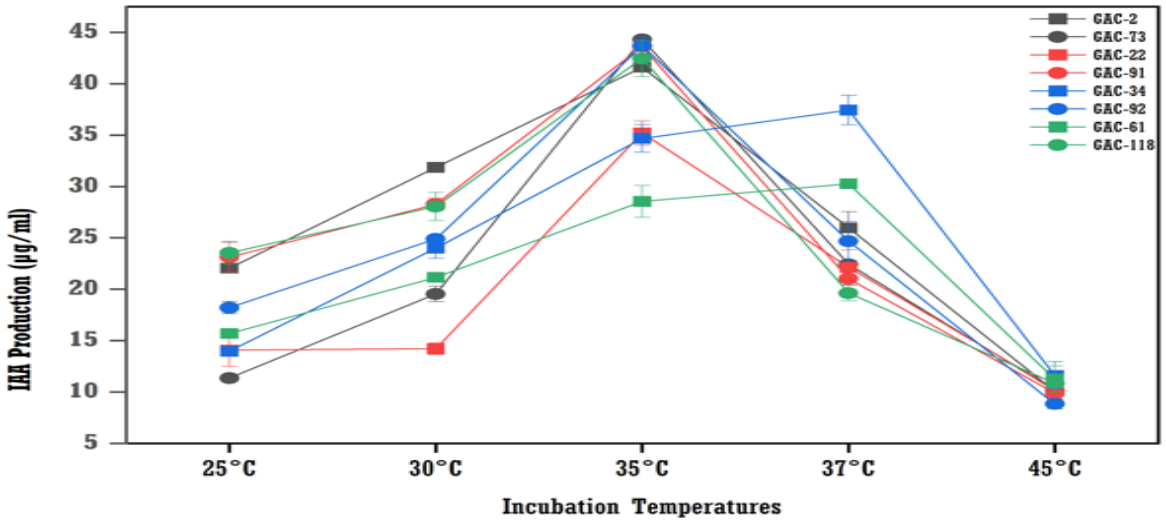

Figure 12.Effect of different temperatures on IAA production by bacterial isolates (means (n=3) ± standard deviation).

4.3.5. Effect on carbon sources on IAA production

The most suitable carbon source for the highest production of IAA by bacterial isolates was sucrose, followed by dextrose, glucose, and fructose (Figure 13) (Appendix 6). The GAC-2 isolate produced the highest amount of IAA at 45.28 µg/ml with sucrose, followed by the GAC-118 and GAC-91 isolates, which reached 44.89 µg/ml and 44.01 µg/ml, respectively. On the other hand, the GAC-118, GAC-2, GAC-92, and GAC-91 produced higher IAA (28.13 µg/ml, 27.35 µg/ml, 26.38 µg/ml, 26.35 µg/ml) under dextrose as a source of carbon. The use of glucose and fructose as carbon sources in the medium for all isolates showed the lowest IAA production.

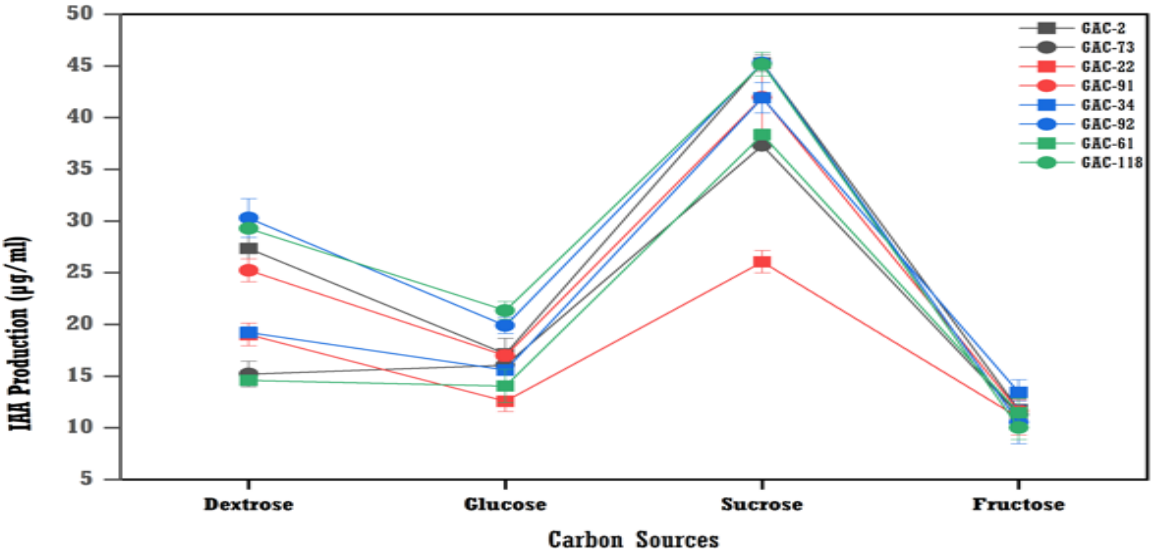

Figure 13.Effect of carbon sources on IAA production by bacterial isolates (means (n=3) ± standard deviation).

4.3.6. Effect of nitrogen sources on IAA production

Results obtained showed that tryptone was the best nitrogen source for the maximum production of IAA for tested bacterial isolates, followed by yeast extract, beef extract, and peptone (Figure 14) (Appendix 7). The optimum production of IAA was obtained in tryptone media, which was 9.23 µg/ml by the GAC-91 isolate, followed by the GAC-22 and GAC-2 isolates with 9.01 µg/ml and 8.58 µg/ml, respectively. The lowest IAA production was obtained at 2.81 µg/ml in peptone media for the GAC-34 isolate.

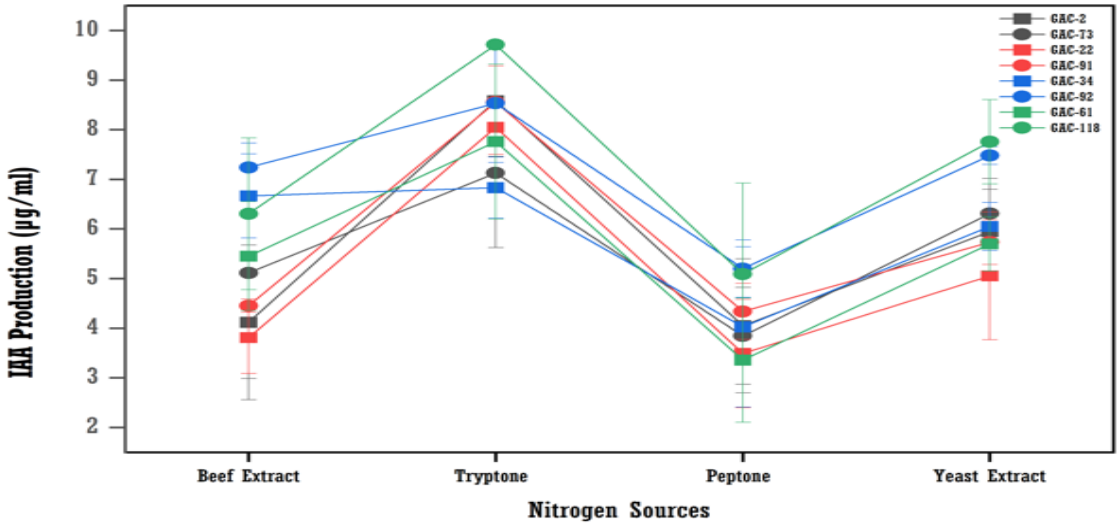

Figure 14. Effect of nitrogen sources on IAA production by bacterial isolates (means (n=3) ± standard deviation).

4.4. Screening of IAA-Produced Isolates for their Plant Growth Promoting Traits

4.4.1. Phosphate solubilization

Two Bacillus isolates (GAC-2 and GAC-73) exhibited a clear halo zone of phosphate solubilization on Pikovaskaya's agar plate. GAC-2 isolate showed a 4.5 mm solubilization zone, and GAC-73 showed a 5 mm solubilization zone. Out of six *Pseudomonas* isolates, four isolates were able to form a clear halo zone of phosphate solubilization on Pikovaskaya's agar plate, and two isolates, GAC-34 and GAC-92, did not form a clear halo zone on Pikovaskaya's agar plate. Among them, GAC-118 isolate detected the highest clear zone at 11.00 mm, followed by GAC-91 (8.5 mm), GAC-22 (4.8 mm), and GAC-61 (4.5 mm) (Table 7) (Figure 15).

4.4.2. Nitrogen fixation

After incubation time, three isolates (GAC-91, GAC-73, and GAC-61) were observed for nitrogen fixation by producing a significant amount of ammonia into the medium by changing the color of the medium from greenish blue to blue distinctly. The other five isolates (GAC-2, GAC-22, GAC-34, GAC-92, and GAC-118) were observed for nitrogen fixation by changing the color of the medium from greenish blue to yellow as acid

production because many nitrogen-fixing bacteria isolates produce acids as a result of the utilization of sugars (Table 7) (Figure 15).

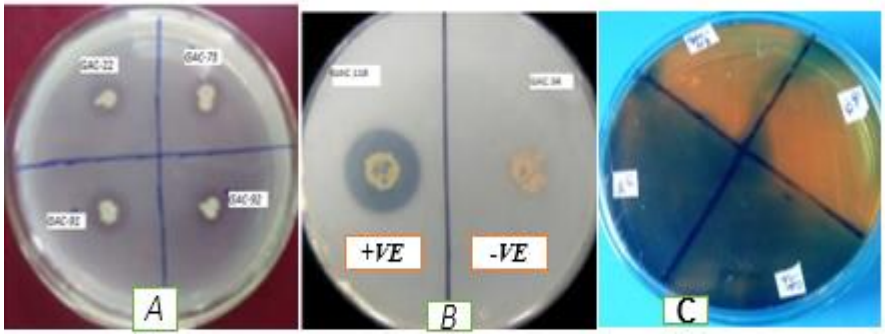

Figure 15. Potentials of bacterial isolates for their plant growth-promoting traits. **(**A**)**: GAC-22, GAC-73, GAC-61, and GAC-92 were positive for phosphate solubilization; **(**B**)**: GAC-118 positive and GAC-34 negative result for phosphate solubilization; and **(**C**)**: All labeled isolates were positive for nitrogen fixation by forming dark blue/yellow color.

Table 7. Properties of bacterial isolates characterized for their plant growth promoting traits.

Isolates Code	**PGPR properties of Isolates**	
	P-Solubilization width of clear zone(mm)	**Nitrogen fixation ability**
GAC-2	4	+
GAC-22	4.8	+
GAC-34	-	+
GAC-73	5	+
GAC-61	4.5	+
GAC-91	8.5	+
GAC-92	-	+
GAC-118	11	+
+= character present, - = character absent		

4.5. Molecular Analysis of Genes

Based on the PCR amplification, only 12 isolates (GAC-2, GAC-117, GAC-73, GAC-22, GAC-118, GAC-30, GAC-42, GAC-59, GAC-82, GAC-88, GAC-104, and GAC-106) possessed the ipdC gene. An 1170 bp DNA fragment found on the gel electrophoresis served as confirmation of this (Figure 16). The product of the expected size (1170 bp) indicated the existence of an IAA-related gene in these isolated bacteria. The ipdC gene was absent from the other bacterial isolates.

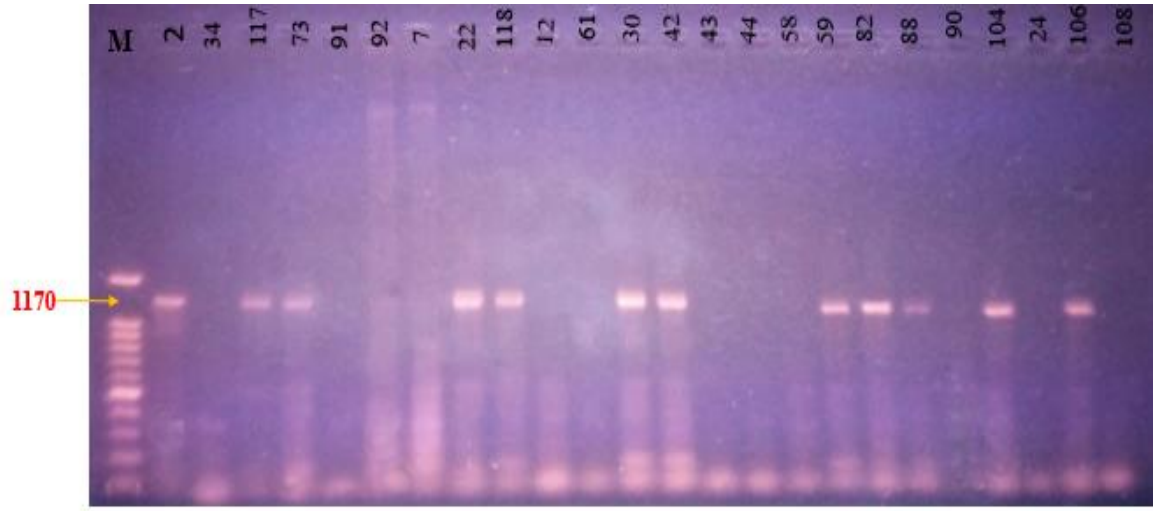

Figure 16. Gel electrophoresis of ipdC (1170 bp) gene amplified by PCR.

*Lane name with respective isolates; **M**-(100 bp ladder), 2–(GAC-2), 34-(GAC-34), 117-(GAC-117), 73-(GAC-73), 91-(GAC-91), 92-(GAC-92), 7-(GAC-7), 22-(GAC-22), 118-(GAC-118), 12-(GAC-12), 61-(GAC-61), 30-(GAC-30), 42-(GAC-42), 43-(GAC-43), 44-(GAC-44), 58-(GAC-58), 59-(GAC-59), 82-(GAC-82), 88-(GAC-88), 90-(GAC-90), 104-(GAC-104), 24-(GAC-24), 106-(GAC-106), and 108-(GAC-108).*

PCR amplification targeting the nifK gene revealed that 10 isolates (GAC-34, GAC-91, GAC-92, GAC-118, GAC-61, GAC-43, GAC-59, GAC-88, GAC-90, and GAC-105) produced an amplified fragment of approximately 360 bp, indicating the presence of the nifK gene (Figure 17). In contrast, the nifK gene was not detected in the remaining isolates. These findings confirmed that the isolates carrying the nifK gene possessed the ability to fix nitrogen.

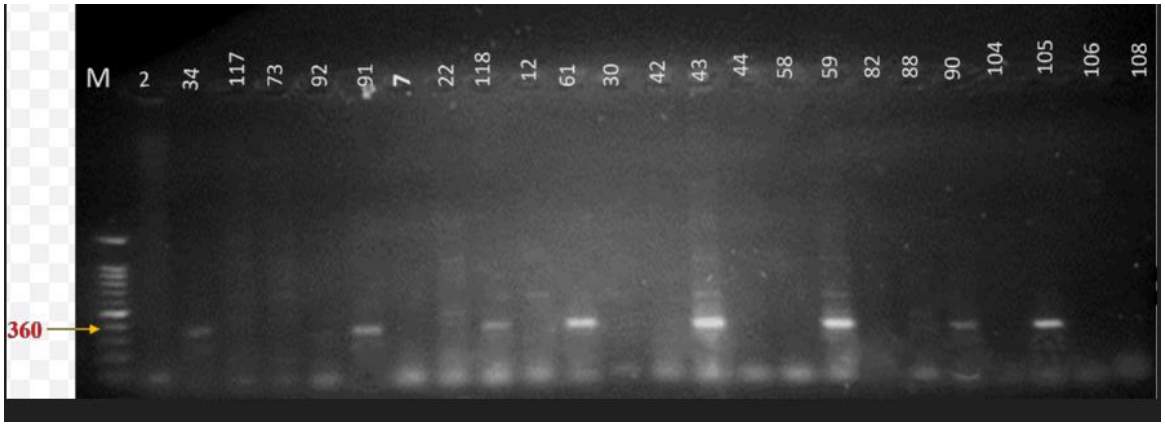

Figure 17. Gel electrophoresis of nifK (360 bp) gene amplified by PCR.

Lane name with respective isolates; ***M****-(100 bp ladder), 2–(GAC-2), 34-(GAC-34), 117-(GAC-117), 73-(GAC-73), 92-(GAC-92), 91-(GAC-91), 7-(GAC-7), 22-(GAC-22), 118-(GAC-118), 12-(GAC-12), 61-(GAC-61), 30-(GAC-30), 42-(GAC-42), 43-(GAC-43), 44-(GAC-44), 58-(GAC-58), 59-(GAC-59), 82-(GAC-82), 88-(GAC-88), 90-(GAC-90), 104-(GAC-104), 105-(GAC-105), 106-(GAC-106), and 108-(GAC-108).*

The results from the nifH gene PCR amplification of the isolates revealed that only 8 isolates (GAC-34, GAC-118, GAC-73, GAC-91, GAC-7, GAC-117, GAC-30, and GAC-42) displayed successful amplification of the nifH gene (Appendix 10). This was evidenced by observing the presence of DNA fragments of the expected size (around 300 bp) in the gel electrophoresis analysis. These findings suggest that these particular bacterial isolates harbor the genetic material responsible for nitrogen fixation. Conversely, none of the other isolated samples showed any presence of the nifK gene.

4.6. Greenhouse Evaluation of IAA Produced Isolates for Growth Promotion

In the greenhouse experiment, the seedlings of chickpea (41209) accession were carefully uprooted after 45 days of sowing, and their plant growth parameters were measured (Table 8) (Figure 18). It was observed that all eight isolates that exhibited higher levels of IAA showed a significant enhancement in both above-ground and below-ground growth parameters of the chickpea 41209 accession compared to the un-inoculated control. However, the results of the greenhouse study revealed that all isolates had a statistically significant increase ($p \leq 0.05$) in chickpea growth compared to the control group.

Data regarding plant shoot length of chickpea (41209) accession exhibited that all isolates had a significant positive effect on the shoot height compared to the un-inoculated control. Among the isolates, the treatment of GAC-118 resulted in the maximum increase in shoot height, reaching 35.867, which corresponds to an 84% increase compared to the control. Additionally, the isolates GAC-91 and GAC-2 exhibited significant increases in shoot height, with values of 32.710 (68% increase) and 31.220 (60% increase) respectively, compared to the un-inoculated control. While the other isolates also led to significant increases in plant shoot height compared to the control, their potential was relatively lower compared to the three isolates mentioned earlier. Notably, the GAC-22 isolate resulted in a decrease in shoot height, with a reduction of 35% (26.367) compared to the un-inoculated control.

The results demonstrated that all isolates had a significant positive increase on the plant shoot fresh weight of the chickpea (41209) accession compared to the un-inoculated control group. Three isolates, namely GAC-118, GAC-91, and GAC-2, showed a significant increase in plant shoot fresh weight compared to the control. The GAC-118 isolate resulted in the highest increase, with a 253% (8.373) increase in plant shoot fresh weight. Similarly, the GAC-91 and GAC-2 isolates exhibited significant increases of 209% (7.327) and 206% (7.263), respectively, compared to the non-treated control. Conversely, the GAC-22 isolate led to a decrease in plant shoot fresh weight compared to the un-inoculated control.

Furthermore, three isolates (GAC-118, GAC-91, and GAC-2) demonstrated a significant increase in plant shoot dry weight. The GAC-118 treatment exhibited the highest significant increase in plant shoot dry weight, with a 277% (4.190) increase compared to the un-inoculated control. Similarly, the GAC-91 and GAC-2 isolates showed significant increases of 250% (3.883) and 239% (3.767), respectively, in plant shoot dry weight. On the other hand, the GAC-92 and GAC-22 isolates resulted in a decrease in shoot dry weight compared to the un-inoculated control. In general, the results indicate that most isolates had a positive effect on both plant shoot fresh weight and plant shoot dry weight, with three isolates (GAC-118, GAC-91, and GAC-2) showing the most significant improvements in both parameters. However, the GAC-22 and GAC-92 isolates exhibited a decrease in shoot dry weight compared to the control.

In this study, all isolates had a significant positive effect on the root length of the chickpea (41209) accession compared to the un-inoculated control group. The highest increase in root length was observed with the GAC-118 isolate, resulting in a 108% increase (25.500) compared to the control. This was followed by the GAC-91 and GAC-2 isolates, which showed increases of 91% (23.333) and 84% (22.517), respectively, over the un-inoculated control. On the other hand, the GAC-22 isolate resulted in a decrease in root length by 38% (16.833) compared to the un-inoculated control.

The data from chickpea accession (41209) revealed that three isolates, namely GAC-61, GAC-73, and GAC-188, showed a significant increase in root fresh weight compared to their respective un-inoculated controls. GAC-61 exhibited the highest increase at 144% (4.360), followed by GAC-73 at 131% (4.127), and GAC-188 at 125% (4.013). The maximum increase in root fresh weight was observed with GAC-61 inoculation, while the minimum increase was seen with GAC-22 at 57% over the un-inoculated control. Significant increases in root dry weight were observed with different isolates. GAC-92 showed the highest increase at 273%, followed by GAC-61 at 231% and GAC-73 at 228% compared to other isolates and the un-inoculated control. However, GAC-22 resulted in a decreased root length by 62% compared to the un-inoculated control. This study demonstrated that all the tested IAA-producing bacterial isolates significantly enhanced the shoot height, root length, and fresh and dry weight of both shoot and root seedlings.

Figure 18. Effects of plant growth-promoting potential of highest IAA-produced isolates and control plants after 45 days of growth on pot culture in the greenhouse. **(**A): GAC-2 isolate; (B): GAC-118 isolate; (C): GAC-92 isolate; (D): control group for plant shoot height; (E): greenhouse arrangement in CRD; (F): GAC-91 isolate; (G): GAC-118 isolate; (H):GAC-92 isolate; and (I): control group for plant growth.

Table 8.The mean separation analysis results of each IAA producing bacterial isolates on different growth parameters in chickpea under greenhouse condition.

Isolates code	Above ground growth parameter			Below ground growth parameter		
	SH (cm)	SFW (g)	SDW (g)	RL (cm)	RFW (g)	RDW (g)
GAC-2	31.220^{bc}	7.263^{b}	3.767^{a}	22.517^{cb}	3.497^{bac}	2.073^{a}
GAC-22	26.367^{d}	3.410^{e}	1.487^{cd}	16.833^{d}	2.807^{c}	1.113^{bc}
GAC-34	29.567^{bcd}	5.660^{c}	2.380^{b}	21.833^{cb}	3.367^{bc}	1.897^{ba}
GAC-61	29.317^{bcd}	4.457^{d}	2.163^{cb}	21.033^{c}	4.360^{a}	2.277^{a}
GAC-73	29.167^{bcd}	4.310^{d}	2.067^{cb}	18.600^{d}	4.127^{ba}	2.250^{a}
GAC-91	32.710^{ba}	7.327^{b}	3.883^{a}	23.333^{b}	3.620^{bac}	2.197^{a}
GAC-92	28.400^{cd}	4.190^{ed}	1.880^{cb}	17.127^{d}	3.320^{bc}	2.563^{a}
GAC-118	35.867^{a}	8.373^{a}	4.190^{a}	25.500^{a}	4.013^{ba}	2.173^{a}
Control (cm)	19.463^{e}	2.370^{f}	1.110^{d}	12.240^{e}	1.787^{d}	0.687^{c}
CV	8.591	9.519	17.289	5.6783	16.516	25.939
LSD (5%)	4.291	0.859	0.756	1.938	0.973	0.852

Means in a column followed by same superscript letters are not significantly different at $P \leq 0.05$; SH = Shoot Height; SFW = Shoot Fresh Weight; SDW = Shoot Dry Weight; RL = Root Length; RFW = Root Fresh Weight: RDW = Root Dry Weight; LSD= Least significant differences of means (5% level); CV= Coefficients of variation.

Table 9 displays the results of the ANOVA analysis conducted on chickpea rhizosphere bacteria that produce IAA to evaluate their impact on various chickpea growth-related parameters. These parameters include plant shoot height, shoot fresh and dry weight, root length, root fresh and dry weight. The ANOVA test demonstrated that the mean values of all agronomic parameters were significantly increased ($p \leq 0.05$) when compared to the un-inoculated control.

Table 9.The effect of IAA producing bacterial isolates inoculation variance on different growth parameters in chickpea under greenhouse condition. Values are means ± S.D.

	Above ground growth parameter			**Below ground growth parameter**		
Isolates code	**SH (cm)**	**SFW (g)**	**SDW (g)**	**RL (cm)**	**RFW (g)**	**RDW (g)**
GAC-2	31.220±2.368	7.26±0.451	3.767±1.079	22.517±1.075	3.497±0.542	2.073±0.657
GAC-22	26.367±1.11	3.41±0.308	1.487±0.445	16.833±0.585	2.806±0.930	1.113±0.225
GAC-34	29.567±3.093	5.66±1.066	2.380±0.178	21.833±1.204	3.367±0.351	1.896±0.335
GAC-61	29.317±2.004	4.46±0.386	2.163±0.155	21.033±0.680	4.360±0.815	2.277±0.342
GAC-73	29.167±2.560	4.31±0.419	2.067±0.221	18.600±2.007	4.127±0.499	2.250±0.053
GAC-91	32.710±2.193	7.33±0.495	3.883±0.021	23.33±1.528	3.620±0.720	2.197±0.288
GAC-92	28.400±4.851	4.19±0.066	1.880±0.105	17.126±0.424	3.320±0.030	2.563±1.172
GAC-118	35.867±0.550	8.373±0.41	4.190±0.429	25.500±0.500	4.013±0.035	2.173±0.153
Control	19.463±0.843	2.37±0.285	1.110±0.289	12.240±1.132	1.787±0.420	0.687±0.163
DF	26	26	26	26	26	26
LSD (5%)	4.291	0.859	0.756	1.938	0.973	0.852
P	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P <0.001	P < 0.001

SH = Shoot Height; SFW = Shoot Fresh Weight; SDW = Shoot Dry Weight; RL = Root Length;
RFW = Root Fresh Weight: RDW = Root Dry Weight; LSD= Least significant differences of means (5% level).

In this finding, a positive correlation was found between the different agronomic parameters, such as shoot height, shoot fresh and dry weight, root length, and root fresh and dry weight, according to the results of the Pearson correlation coefficient analysis (Table 10). Pearson correlation coefficient values between (0 and ±0.29) indicate a weak positive (negative)), (± 0.3 to ± 0.69) indicate a moderate positive (negative) and (± 0.7 to ±1.0 indicate a strong positive (negative) linear relationship. In this case, the positive correlation between the agronomic parameters can be classified as strong and moderate, based on the reference values provided. The study's growth-promoting rhizobacteria were able to consistently and successfully influence all growth-related traits, including the

production of IAA, as indicated by the strong and moderate positive correlations found among the agronomic parameters.

Table 10. Pearson correlation coefficient comparisons for all agronomical parameters (plant shoot height, shoot fresh and shoot dry weight, plant root length, root fresh and root dry weight).

Correlations						
	SH	**SFW**	**SDW**	**RL**	**RFW**	**RDW**
SH						
SFW	0.820**					
SDW	0.759**	0.906**				
RL	0.866**	0.911**	0.841**			
RFW	0.575**	0.461**	0.448**	0.579**		
RDW	0.655**	0.439*	0.501**	0.538**	0.578**	

** Correlation is moderate significant at 0.05; ** indicates correlation is strongly significant at the 0.01. SH = Shoot Height; SFW = Shoot Fresh Weight; SDW = Shoot Dry Weight; RL = Root Length; RFW = Root Fresh Weight: RDW = Root Dry Weight*

5. DISCUSSION

Phytohormones, which are plant growth regulators, have a significant impact on plant growth (Maharana, 2019; Ramadhani *et al*., 2020). Many species present in the rhizosphere can synthesize IAA (Imran *et al.*, 2021), a vital signaling molecule that regulates plant processes such as growth, cell activities, division, and gene expression (Lakshmanan *et al*., 2022). In this study, a total of 118 bacterial isolates were obtained, and among them, 74 isolates were isolated from the soil rhizosphere and 44 isolates were from the root nodule sample. All 27 isolates were found to produce IAA in the range of 2.15 µg/ml to 26.47 µg/ml when grown in medium containing tryptophan at a concentration of 500 µg/ml. Similar findings have been reported in previous studies, where various strains of bacteria produced IAA in the range of 20 mg/l to 90 mg/l in culture media containing tryptophan at a concentration of 0.5g/l (Kyaw *et al*., 2019). This finding also aligns with previous studies that have found IAA-producing organisms to be predominantly gram-negative, with a few gram-positive strains belonging to the *Bacillus* genus (Mohite, 2013; Bhutani *et al*., 2018; Maharana, 2019). Other studies have reported the production of phytohormones by specific strains of *Pseudomonas putida UB1* (Bharucha *et al*., 2013)*,* Rhizobium *leguminosarum* strains (Shoukry *et al.*, 2018)*, Bacillus siamensis* (Widawati & Suliasih, 2020)*,* and other *Pseudomonas* species (Malik & Sindhu, 2011) in chemically defined media. It is known that IAA production can vary among different bacterial species or strains of the same species. Additionally, factors such as microbial species, strains, culture conditions, substrate availability, and growth stage can influence IAA production (Mohite, 2013; Kyaw *et al.,* 2019; Lebrazi *et al*., 2020; Widawati & Suliasih, 2020).

Microscopic and colony morphology analyses were performed on the bacterial isolates, following the methodologies outlined in the studies conducted by Babiye Birhanu *et al.* (2021). On their respective medium plates, the isolates showed features such as small to medium size, red and yellowish white color, and an off-white (creamy white) appearance around the colonies. The biochemical identification of the isolates was conducted following the methods described by Babiye Birhanu *et al.* (2021) and Yonas Tassew & Samuel Sahile (2017). By comparing the biochemical results with Bergey's Manual of Systematic Bacteriology Studies (Bergey, 2008), two isolates (GAC-2 and GAC-73) were identified as *Bacillus* species, while the remaining six isolates were classified as *Pseudomonas* species.

L-tryptophan, which is an essential amino acid, plays a vital role in various biological processes in plants (Bhutani *et al.*, 2018). This study showed that there was a gradual increase in IAA production as the concentration of L-tryptophan increased up to 500 µg/ml. The highest production of IAA was observed when the L-tryptophan concentration was 500 µg/ml for all the bacterial isolates. These findings align with a study by Shoukry *et al*. (2018), where it was reported that the production of IAA from bacterial strains increased with increasing concentrations of L-tryptophan up to 5 mg/ml. Similarly, Bharucha *et al.* (2013) reported that the production of IAA from *Pseudomonas putida UB1* strains increased with increasing concentrations of L-tryptophan up to 200 µg/ml.

According to earlier research by Bhutani *et al*. (2018), the incubation period for the synthesis of IAA can range from a few days to a full day. The production of IAA by the isolates started after 24 hours and reached its highest level after 72 hours of incubation. After that, bacterial growth gradually decreased. These findings align with previous research by Bhutani *et al*. (2018) and Shoukry *et al.* (2018), which indicated that the optimal incubation period for maximum IAA production ranges from 48 to 72 hours. However, the findings of Sadhana *et al.* (2017) and Widawati & Suliasih (2020) differed, as they reported that an incubation period of 96 to 120 hours was optimal for IAA production. The decrease in IAA content after the stationary phase (72 hours) could be attributed to the release of IAA degrading enzymes such as IAA oxidase and IAA peroxidase, as mentioned in previous studies by Shoukry *et al*. (2018).

The pH of the production media plays a crucial role in the growth and metabolic activity of IAA-producing organisms. This study revealed that the highest production of IAA occurred when the media had a pH of 7, followed by pH 8. These findings was consistent with previous studies conducted by Harikrishnan *et al.* (2014); Shoukry *et al*. (2018); Widawati & Suliasih (2020), which also reported the highest IAA production at pH 7 and pH 8. Bharucha *et al.*, (2013) demonstrated that *Pseudomonas putida UB-1* showed maximum IAA production at pH 7.5, whereas Chandra *et al*. (2018) suggested that IAA production varied between pH 5-9, with the highest production observed at pH 9. Temperature is one of the factors influencing enzymes activity involved in IAA biosynthesis. Among the eight isolates tested, six isolates exhibited the highest IAA production at 35°C, while the

remaining two isolates showed maximum production at 37°C. These findings have been consistent with previous studies conducted by Chandra *et al.* (2018); Shoukry *et al.* (2018); Widawati & Suliasih (2020).

The carbon source plays a crucial role in the production of metabolites by microorganisms. Among these carbon sources, sucrose was found to stimulate the highest IAA production, followed by dextrose, glucose, and fructose for all isolates. Conversely, the use of fructose resulted in the lowest IAA production. The superior performance of sucrose as a carbon source for IAA production could be attributed to its better utilization by bacteria during growth compared to other sugars. Thus, sucrose was identified as the most efficient carbon source for IAA production. Previous studies by Chandra *et al*. (2018) and Chouhan *et al*. (2022) have also reported similar observations, highlighting the influence of individual carbon sources on IAA production. Additionally, studies conducted by Bharucha *et al*. (2013) and Sadhana *et al*. (2017) have demonstrated that bacteria grown on media supplemented with 1% sucrose exhibit the highest IAA production. The presence of nitrogen in the cell is crucial as it is a fundamental element in proteins, nucleic acids, and cell walls. The choice of nitrogen source in the culture medium directly impacts the growth and metabolism of microorganisms (Chouhan *et al.*, 2022). This finding revealed that tryptone medium showed the highest IAA production, followed by yeast extract, beef extract, and peptone. The GAC-91 isolate achieved the highest IAA production of 9.23 µg/ml in tryptone medium. These findings align with previous studies by Widawati & Suliasih, (2020), who found that *Bacillus siamensis* isolated from peat showed maximum IAA production in media containing tryptone as the nitrogen source. Similarly, Sadhana *et al*. (2017)and Bhutani *et al*. (2018) reported that the choice of nitrogen source in the medium influenced both cell growth and IAA production.

In our current study, we characterized the ability of bacterial isolates from the chickpea rhizosphere to exhibit plant growth-promoting traits, such as phosphate solubilization and nitrogen fixation. However, the performance of these traits varied among the bacterial isolates when tested in vitro. The nitrogen fixation ability is an important criterion in the selection of potential PGPR. The bacterial isolates were grown on G-NFM medium, indicating their ability to fix nitrogen (Imran *et al*., 2021). In this finding, three isolates exhibited nitrogen fixation by producing a significant amount of ammonia in the medium,

resulting in a distinct color change from greenish blue to dark blue. On the other hand, five isolates exhibited nitrogen fixation by changing the medium color from greenish blue to yellow, indicating acid production. This is because many nitrogen-fixing bacteria produce acids as a result of sugar utilization. The alteration in color of the medium supplemented with bromothymol blue from greenish blue to dark blue suggests the production of ammonium. The presence of ammonia-producing bacteria indicates the occurrence of ammonification in the rhizosphere soil, which can increase the pH of the medium. Consequently, the pH increase resulted in the color changes observed (Sulistiyani & Meliah, 2017). These findings are consistent with the findings of Zerihun Tsegaye *et al.* (2019), who characterized nitrogen-fixing bacteria associated with Sorghum (*Sorghum bicolor*), and Lwin *et al*. (2012), who screened IAA-producing *Bacillus* and *Serratia* bacterial species for nitrogen fixation abilities from various rhizosphere soils in Mandalay. The color changes from greenish blue to yellow indicate the presence of acid-producing nitrogen-fixing bacteria.

Even though phosphorus is abundant in agricultural soils, plants frequently cannot access it because of its low solubility (Imran *et al*., 2021). Because of this restriction, researchers studying agricultural microbiology are investigating the possibility of rhizobacteria to solubilize insoluble phosphates, which can improve plant growth and yield by making more phosphorus available. Using phosphate-solubilizing PGPR as bio-inoculants is one of the biotechnological approaches in sustainable agriculture (Zerihun Tsegaye *et al.*, 2019; Babiye Birhanu *et al.*, 2021). In this study, most of isolates showed a halo zone and exhibited higher rates of phosphate solubilization and rest isolates do not solubilized phosphate (Figure 15 A). The widest halo zone was observed with bacterial isolate GAC-118 (11 mm). These findings are consistent with previous research that has identified various species of *Rhizobium, Azotobacter, Pseudomonas, and Bacillus* as capable of solubilizing phosphate (Yadav *et al*., 2014; Kyaw *et al*., 2019; Babiye Birhanu *et al*., 2021; Imran *et al*., 2021; Pathak *et al.*, 2021).

Numerous investigations have demonstrated that even minute concentrations of IAA released by PGPR can control plant growth, particularly by encouraging cell division and elongation and fostering plant resistance to disease (Xiaobo *et al.*, 2022). PGPR synthesizes IAA through various pathways, including the indole-3-pyruvic acid pathway, the indole-3-

acetamide pathway, the tryptophan side chain pathway, the tryptamine pathway, and the indole-3-acetonitrile pathway, all of which depend on the amino acid tryptophan (Keswani *et al*., 2020). The IPyA pathway is the most important pathway for IAA biosynthesis in bacteria. It has been found in a wide range of bacteria, including *Bacillus sp., Enterobacter sp., Azospirillum sp., Rhizobium sp., Pseudomonas sp.,* and *Arthrobacter sp*. (Moreno *et al*., 2015; Zhang *et al.*, 2021; Shah *et al.*, 2022). The ipdC gene is essential for the synthesis of indole-3-pyruvate in the IPyA pathway. In this pathway, tryptophan is oxidatively deaminated to indole-3-pyruvate, which is then converted to indole-3-acetaldehyde by the action of indole-3-pyruvate decarboxylase (encoded by ipdC) and then to IAA (Xiaobo *et al.*, 2022). A study by Zhang *et al*. (2021) found that the ipdC gene has been identified in the IPyA pathway of *Pseudomonas putida, Bacillus,* and *Azospirillum brasilense* microorganisms. Therefore, mining for functional genes is a valuable tool for improving plant growth-promoting rhizobacteria (PGPR) strains. It can be used to strengthen research on the growth-promoting characteristics of strains, supplement the metabolism of strains, and improve the application efficiency of PGPR in plants (Xiaobo *et al*., 2022).

Nitrogen fixation is a biological process facilitated by the nitrogenase enzyme, which is encoded by various **nif** genes including nifH, nifD, and nifK (Imran *et al.*, 2021). Among these, nifH gene, which encodes the nitrogenase reductase subunit, is widely sequenced marker and used as a reliable conserved gene to identify nitrogen-fixing bacteria (Idris *et al*., 2015; Imran *et al.*, 2021). This study confirmed the molecular approach of analyzing nifH and nifK genes in the selected cultures, enabling the identification of these beneficial microorganisms. These findings align with Upadhyay *et al*. (2009); Amara *et al*. (2015), Sulistiyani & Meliah, (2017) and Imran *et al.,* (2021), that have documented the presence of nifH genes in various rhizosphere bacteria species extracted from nodules of different leguminous plants. The nifH gene is recognized as the most well-known functional gene and serves as a valuable tool in validating the potential for nitrogen fixation (Idris *et al*., 2015). These findings align with prior research conducted by Dai *et al.* (2014), which demonstrated the presence of nifK genes in *Methylococcus capsulatus, Betaproteobacteria, Herbaspirillum,* and *Burkholderia* genera isolated from acid mine drainage environments.

The ability of bacteria to synthesize IAA is considered as a valuable tool for identifying beneficial microorganisms, as IAA-producing bacteria have a significant impact on plant growth (Mohite, 2013). The results clearly demonstrate that the production of IAA by these bacterial isolates played a significant role in the observed differences between the treated and control groups, with statistical significance ($P \leq 0.05$). Similar studies have previously been conducted with bacterial inoculation in various crops, including wheat (Yonas Tassew & Samuel Sahile, 2017), maize (Lwin *et al.*, 2012), sorghum (Babiye Birhanu, 2022), mung (Anjum *et al.*, 2011), coffee (Kunwar *et al*., 2018), and chickpea (Yadav *et al.*, 2014).

Data regarding plant shoot height, the treatment with GAC-118 showed the highest height of 35.867 cm, when compared to un-inoculated control (19.463 cm). These findings align with several other studies that have reported an increase in plant shoot height with the use of selected isolates (Harikrishnan *et al*., 2014; Bhutani *et al.*, 2018; Babiye Birhanu, 2022). Furthermore, previous reports have indicated that inoculation with IAA-producing bacteria also leads to an increase in shoot height compared to un-inoculated control group (Ahmed & Hasnain, 2010; Waday *et al.* 2022). In comparison to the uninoculated control, all of the isolates exhibited a statistically significant increase in shoot fresh weight as compared to the uninoculated control. However, the other isolates also significantly increased the shoot fresh weight of chickpea plants compared to the control, but their potential was relatively lower compared to the above mentioned three isolates. This finding is consistent with a study conducted by Babiye Birhanu *et al.* (2021), which reported that all selected isolates showed an increase in plant shoot height compared to the control. Additionally, a study by Waday *et al.* (2022) demonstrated that isolates promoting an increase in plant shoot height also led to an increase in shoot fresh weight, which supports the results of this current study. The selected isolates significantly increased the shoot dry weight of chickpea seedlings. It is likely that the potential of bacterial isolates to promote above-ground plant biomass growth varies based on their genomic characteristics. It's important to consider factors such as environmental conditions, soil type, the level of IAA produced by both the plant and the PGPR, as well as the conditions in the greenhouse. These factors can influence the growth-promoting potential of bacterial isolates on the shoot dry weight, as mentioned in the study conducted by Babiye Birhanu (2022). In our current study, all the tested bacterial isolates producing IAA demonstrated an increase in plant shoot dry weight to varying degrees, which aligns with findings reported by Yadav *et al*. (2014).

In the present study, a significance increase in plant root length was observed when different treatments of bacterial isolates were applied compared to the un-inoculated control. Similar to this findings, previous studies conducted by Idris *et al*. (2009) and Babiye Birhanu *et al.* (2021) also reported significant increases in root length due to the presence of isolates with various potentials. Additionally, Mohite (2013) observed a considerable enhancement in both root and shoot length of wheat plants when treated with IAA-producing bacterial isolates compared to the untreated control. In terms of root fresh weight, isolates exhibited a significant increase as compared to the un-inoculated control. Significant increases in root dry weight were also observed in response to different isolates. In this study, the isolates that significantly increased root length did not necessarily lead to an increase in root fresh and dry weight. This discrepancy could be attributed to various factors, including the environmental conditions favoring the growth of these beneficial bacteria (PGPR) or variations in the isolates' potential to colonize the roots and enhance root fresh and dry weight. In contrast to the findings of this study, a study conducted by Yadav *et al*. (2014) reported that all the isolates that increased root length also led to an increase in both root fresh and dry weight, contradicting our results.

The results of the analysis of variance (ANOVA) test indicated that, in comparison to the uninoculated control, the mean values of all agronomic parameters were significantly higher ($p \leq 0.05$). The different PGPR chickpea rhizosphere bacteria tested exhibited significant differences in their growth-promoting potential, which correlated with their ability to produce IAA, as supported by the findings of Babiye Birhnau (2022). Furthermore, there was a positive correlation between all agronomic parameters, as indicated by strong and moderate values of the Pearson correlation coefficient. These results align with the findings of Babiye Birhanu *et al.* (2021) and Asmamaw Menelih *et al*.,(2022). However, it should be noted that in a study by Anjum *et al.* (2011), a negative correlation was observed between plant height and root length, as well as fresh weight, which contradicts the findings of the current study. Taken together, these studies suggest that the production of IAA is a crucial trait for effective bio-inoculants. One way to lessen the reliance on chemical fertilizers is to promote organic farming practices by inoculating plants with isolates that have the potential to produce IAA.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The study's results showed that there is a varied population of bacteria in the soil around chickpea roots as well as in the nodules on the roots themselves. These bacteria are capable of generating the IAA, which is well known for promoting plant growth. Among the 27 bacterial isolates identified as IAA producers, 8 isolates exhibited notably higher levels of IAA production. These isolates were subjected to detailed characterization, including assessments of their morphology, biochemical traits, and physical parameters, to optimize IAA production. Furthermore, the evaluation of the plant growth promotion traits of the isolated IAA-producing bacteria, such as phosphate solubilization and nitrogen fixation activity, has determined their potential to enhance plant growth. Under greenhouse conditions, the effects of the IAA-producing bacterial isolates on chickpea plant growth demonstrated significant improvements in all plant growth parameters compared to the control group, and molecular techniques were employed to analyze the presence of IAA and nitrogen fixing-related genes. This study represents a significant exploration into IAA production by PGPR isolated from the chickpea rhizosphere, expanding our insights into the role of IAA-producing PGPR in rhizosphere samples. The highest IAA production was observed in *Bacillus* and *Pseudomonas* species cultured in a medium supplemented with 500 µg/ml of L-tryptophan at 35°C, following a 72-hour incubation period. The results are promising to develop a formulation of potentially active plant growth-promoting strains that can promote plant growth and improve crop yields. In the absence of chemical fertilizers, they might also be great substitutes for fostering plant growth, especially in developing nations like Ethiopia. However, more research is required to confirm these potential isolates in detail and in field application, taking into account different soil types and climates, particularly for crops that are significant economically and molecular identification through 16S rRNA sequencing.

6.2. Recommendation

Based on the results of this study, we recommend the following:

- Chickpea rhizosphere isolates obtained from soil samples (GAC-2, GAC-22, and GAC-34) and root nodule samples (GAC-61, GAC-73, GAC-91, GAC-92, and GAC-118) showed excellent plant growth performance and were optimized under different physiological factors and *in vitro* PGP traits. They are hence highly recommended for field trial and ecological competitiveness studies under different Ethiopian soils, different genotypes of chickpea plants, and climatic conditions.

- During this study methods used for characterizing were morphological, cultural, and biochemical. Hence, such kinds of study must be substantiated by molecular methods using 16S rRNA sequence analysis so as to obtain a better understanding of microbial diversity and strain identification.

- To get effective and ecologically adaptive chickpea rhizosphere IAA-producing bacteria, the collection of large samples size from many chickpea-growing areas is recommended.

- The result of the current study indicated that IAA-producing isolates such as GAC-2, GAC-91, and GAC-118 are among the effective isolates that showed a significant enhancement in all agronomical parameters and need to be taken to the field under different agroecological zones as they could be used as efficient biofertilizers in chickpea production systems in Ethiopia.

- Effects of selective inoculation of IAA-produced isolates on chickpea plants significantly enhanced plant growth performance, and hence, such kinds of studies are highly recommended using co-inoculation of efficient IAA-produced isolates and rhizosphere isolates isolated from different crop plants.

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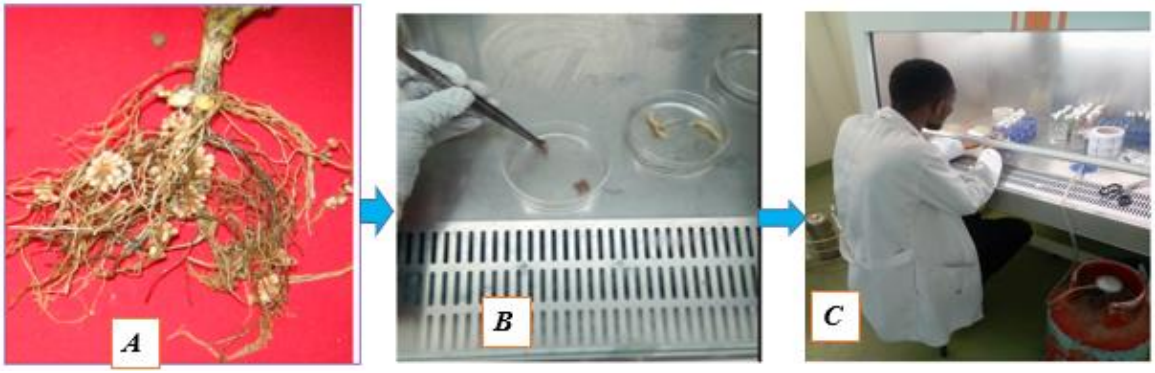
8. APPENDICES

Appendix 1. Chickpea root nodule and soil sample collection from Abuko Woreda.



Appendix 2. Bacteria isolation from root nodule samples: A: collected nodule samples; B: crushing of root nodules under cabinet; and C: inoculation in to prepared media.

Appendix 3. IAA standard curve value

Standard curve	
IAA concentration(µg/ml)	**OD Reading at 530 nm**
0	0.245
10	0.544
20	0.823
30	0.94
40	1.23
50	1.457
60	1.83
70	2.03
80	2.56
90	2.75
100	3.54

Appendix 4. The effect of different pH values on IAA production

Isolates code	PH value				
	5	6	7	8	9
GAC-2	17.06±1.12	38.97±0.43	60.56±1.40	41.83±1.44	23.84±0.78
GAC-22	9.73±0.44	39.45±1.18	43.67±0.96	46.04±0.93	22.36±0.17
GAC-34	13.47±1.33	34.25±1.17	46.830.15±	36.84±1.47	15.14±0.17
GAC-61	19.83±0.89	37.43±0.65	51.20±2.19	39.81±1.33	16.38±0.07
GAC-73	14.52±0.41	40.83±1.04	43.21±1.05	51.48±2.16	10.44±1.72
GAC-91	22.40±1.68	41.41±1.47	61.85±3.43	44.61±1.15	14.65±0.07
GAC-92	16.54±1.43	38.73±1.45	47.10±1.74	44.14±0.60	21.15±0.33
GAC-118	21.83±1.01	38.83±1.01	60.62±1.78	43.95±0.78	14.44±0.01

Appendix 5. The effect of different incubation temperature on IAA production

Isolates code	Temperature value (℃)				
	25℃	30℃	35℃	37℃	45℃
GAC-2	22.06±0.17	31.85±0.17	41.62±0.17	25.97±1.58	10.08±0.55
GAC-22	14.62±1.67	14.75±0.59	36.98±1.24	23.13±1.78	10.62±0.86
GAC-34	11.46±0.21	21.50±1.01	32.18±1.35	34.94±1.45	9.12±1.34
GAC-61	15.10±0.17	22.81±0.21	33.23±2.19	35.66±0.18	8.82±1.79
GAC-73	11.35±0.22	19.54±0.75	44.32±0.20	22.42±0.25	10.15±0.63
GAC-91	24.18±1.52	29.65±0.61	45.89±0.49	21.97±1.17	10.17±0.62
GAC-92	15.70±0.60	22.40±0.09	41.21±0.85	22.18±1.91	6.34±0.51
GAC-118	26.16±1.58	32.54±1.94	52.90±2.52	20.63±1.07	8.25±0.69

Appendix 6. The effect of different carbon sources on IAA production

Isolates code	Carbon sources			
	Dextrose	Glucose	Sucrose	Fructose
GAC-2	27.35±1.86	17.15±1.49	45.28±0.83	11.75±1.04
GAC-22	19.79±1.15	12.98±1.06	27.23±1.15	11.31±1.77
GAC-34	15.93±0.52	12.49±0.15	37.37±1.38	10.47±1.15
GAC-61	12.62±0.16	12.04±1.71	37.71±0.57	9.30±1.50
GAC-73	15.19±1.23	16.06±1.54	37.31±0.15	11.28±0.94
GAC-91	26.35±1.17	17.64±0.61	44.01±3.41	12.01±1.62
GAC-92	26.38±1.78	16.56±0.73	40.55±0.12	7.72±1.96
GAC-118	28.13±0.11	19.75±0.91	44.89±1.22	7.79±1.22

Appendix 7. The effect of different nitrogen sources on IAA production

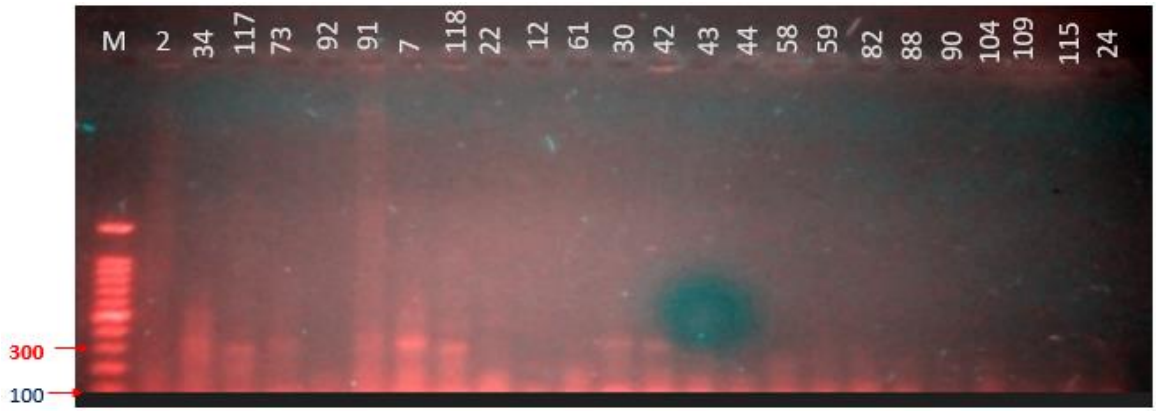
Isolates code	Nitrogen sources			
	Peptone	Beef extract	Tryptone	Yeast extract
GAC-2	4.05±1.35	4.12±1.56	8.58±1.13	5.95±0.85
GAC-22	3.43±1.33	3.82±0.88	9.01±0.67	5.35±1.57
GAC-34	2.81±1.79	5.74±0.94	5.92±0.68	5.06±0.54
GAC-61	3.45±0.97	5.07±0.85	6.87±1.22	5.27±0.43
GAC-73	3.84±0.98	5.11±2.13	7.13±1.50	6.31±0.71
GAC-91	4.47±0.69	4.61±0.16	9.62±0.89	6.18±0.55
GAC-92	4.11±0.64	6.38±0.54	7.81±1.33	6.64±0.20
GAC-118	4.79±1.43	5.74±1.19	8.39±0.05	6.87±0.66

Appendix 8. The effect of incubation period on IAA production

Isolates code	Incubation times (Hours)				
	24	48	72	96	120
GAC-2	12.46±1.85	22.67±1.43	16.86±1.11	7.95±0.85	4.36±0.45
GAC-22	12.11±0.49	24.46±1.42	18.96±0.71	5.35±1.57	3.55±0.01
GAC-34	12.70±0.35	20.78±0.78	23.78±0.63	5.51±1.65	4.44±0.62
GAC-61	10.54±0.67	23.15±0.41	16.65±0.29	7.02±0.78	3.57±0.33
GAC-73	13.68±0.16	22.82±0.08	24.88±0.13	7.31±0.71	4.96±0.59
GAC-91	13.84±0.71	23.11±0.15	16.61±0.55	9.68±0.16	3.67±0.01
GAC-92	12.40±1.49	21.89±0.79	16.89±0.79	8.14±2.32	4.10±0.57
GAC-118	12.61±1.19	23.62±0.87	19.62±0.87	9.37±1.37	3.96±0.56

Appendix 9. The effect of L-tryptophan concentration on IAA production

Isolate code	L-tryptophan concentration (µg/ml)					
	100	200	300	400	500	600
GAC-2	10.33±0.62	16.83±1.32	23.57±1.57	27.16±0.73	34.56±1.27	17.57±1.26
GAC-22	11.11±0.93	12.36±1.99	21.46±1.41	24.96±0.71	32.16±1.49	20.46±0.06
GAC-34	9.55±1.56	14.27±0.58	17.78±2.19	23.78±0.63	27.94±0.06	17.28±1.49
GAC-61	11.04±1.37	11.74±0.94	12.55±0.16	23.05±0.55	31.24±0.79	15.55±1.57
GAC-73	5.18±0.55	17.18±0.55	18.82±1.33	23.32±0.63	30.51±2.12	18.82±1.33
GAC-91	7.84±0.70	17.34±1.41	22.50±0.70	24.11±1.26	33.88±0.32	19.03±1.41
GAC-92	10.89±0.63	19.89±0.63	22.39±1.49	23.39±1.33	32.58±0.81	19.69±0.90
GAC-118	8.11±0.93	20.61±1.64	23.62±0.55	24.62±0.55	33.37±0.86	15.62±0.55



Appendix 10. Gel electrophoresis of nifH (300 bp) gene amplified by PCR.

Lane name with respective isolates; ***M-****(100 bp ladder), 2–(GAC-2), 34-(GAC-34), 117-(GAC-117), 73-(GAC-73), 92-(GAC-92), 91-(GAC-91), 7-(GAC-7), 118-(GAC-118), 22-(GAC-22), 12-(GAC-12), 61-(GAC-61), 30-(GAC-30), 42-(GAC-42), 43-(GAC-43), 44-(GAC-44), 58-(GAC-58), 59-(GAC-59), 82-(GAC-82), 88-(GAC-88), 90-(GAC-90), 104-(GAC-104), 105-(GAC-105), 109-(GAC-109), and 115-(GAC-115).*

Appendix 11. Bacterial treatments applied to chickpea plant growth performance on pot culture in the greenhouse.

Treatments	
Control	Non-inoculated soil-no bacteria
GAC-2	Soil inoculated with GAC-2
GAC-22	Soil inoculated with GAC-22
GAC-34	Soil inoculated with GAC-34
GAC-61	Soil inoculated with GAC-61
GAC-73	Soil inoculated with GAC-73
GAC-91	Soil inoculated with GAC-91
GAC-92	Soil inoculated with GAC-92
GAC-118	Soil inoculated with GAC-118

Appendix 12. Sampling sites/areas of chickpea rhizosphere soil and nodules samples from Gurage Zone-selected woreda.

Sampling sites		Altitude	Locations		Soil pH
Woreda	**Kebele**	**(m.a.s.l)**	**Latitude**	**Longitude**	
	Abuko	1663	8°20'36"N	37°40'70"E	5.77
	Darge	1533	8°24'57"N	37°73'17"E	5.63
	L/Geraba	1882	8°16'31"N	37°48'13"E	6.02
	Fenta	1771	8°16'24"N	37°42'32"E	7.06
	Jejeba	1825	8°15'41"N	37°44'11"E	7.21
Abeshige	Lache	1707	8°18'42"N	37°41'44"E	7.01
	Fite	1567	8°19'13"N	37°36'06"E	6.31
	T/Geraba	1815	8°15'48"N	37°47'15"E	6.43
	Hudada 4	1565	8°21'59"N	37°31'28"E	6.57
	Michele	1749	8°18'53"N	37°44'20"E	5.64
	Borer	1538	8°18'38"N	37°33'10"E	5.89
	K/Kabada	1678	9°12'12"N	36°34'50"E	6.30
	Remuga	1830	9°15'10"N	36°28'44"E	6.20
	Fikadu	1855	9°18'61"N	36°54'24"E	6.38
Kebena	Rekaboka	1819	9°16'67"N	36°44'54"E	6.23
	Ferejete	1730	9°21'29"N	36°66'33"E	6.53
	Lencca	1884	9°19'75"N	37°10'79"E	6.17
	Wosharbe	1907	9°19'54"N	38°74'87"E	6.06
	Zebimola	1867	9°17'28"N	36°84'81"E	6.32
	Negesa	2029	9°19'70"N	38°55'24"E	5.81
	Genbela	1927	9°12'63"N	38°50'74"E	5.93
Sodo	Anati	2045	8°19'79"N	38°56'29"E	5.86
	D/H/Gebeya	1839	8°98'32"N	38°53'56"E	5.25
	Firshi	2003	8°33'73"N	38°53'05"E	5.7
	Goget 1	1803	8°28'56"N	38°23'98"E	6.14
	Goget 2	1823	8°32'79"N	38°11'78"E	6.01
South Sodo	Golbe	1904	8°53'34"N	38°45'06"E	6.30
	Goget 3	1842	8°56'06"N	38°34'12"E	5.97
	Agemsenad	1900	8°20'89"N	38°67'42"E	6.13
	K/Nurena	1901	8°43'13"N	38°56'07"E	5.89