



## Article

# Improving Productivity and Fruit Quality of Acid Lime Trees Using Biofertilizers and Wood Vinegar Under Calcareous Soil Conditions

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**Abstract:** Citrus cultivation in Egypt's calcareous soils faces significant challenges, including a lack of nutrients (particularly iron and phosphorus), soil compact that makes aeration and water absorption difficult, an increase in fungal and bacterial disease such as gummosis, and high salinity that inhibits root growth, resulting in weak growth and low productivity, all of which require an urgent need for solutions. Thus, the current study was conducted over two consecutive seasons 2022-2023 at a private orchard in El Azyma city, El-Beheira governorate, Egypt on acid lime trees (*Citrus aurantifolia* Swingle) grown in sandy soil under drip irrigation system, to ameliorate of calcareous soils through treating these soil by PGPB; plant growth-promoting bacteria (*Bacillus velezensis* and *Cytobacillus firmus*) and wood vinegar additions as well as enhancement lime fruit yield and fruit quality under these conditions. Generally, the results revealed that, wood vinegar treatment attained the highest values concerning fruit yield (54.07 and 60.11 kg/ tree in both seasons, respectively) and the peroxidase activity at the two successive seasons. However, the *Cytobacillus firmus* treatment generated the notable maximum fruit weight and fruit juice percent percentage in both subsequent seasons. Finally, the results showed that adding PGPB reduced leaves total phenols concentration, while increasing the concentrations of proline and ascorbic acid substances. Additionally, during the second season, PGPB ranked second in terms after wood vinegar treatment in increasing the peroxidase enzyme activity, all of these substances were produced by acid lime trees treated by PGPB acts as a primary plant defense system to cope with stresses, when compared to the control. Data obtained reflect different response with varied treatments.

**Key words:** Acid lime, calcareous soil, plant growth promoting bacteria, wood vinegar, plant defense system.

## 1. Introduction

Acid lime (*Citrus aurantifolia* Swingle) is one of the Egypt's economically important citrus fruits, along with sweet orange, mandarin, and lemon. Lime is a multipurpose fruit that provides several health advantages and treatment from a variety of ailments. It is utilized not only in the home, but also in several industrial applications such as cosmetics, medicines, and food processing (**Kurbett and Khyadagi, 2023**).

Preliminary observations regarding private citrus orchard located in El- Beheira Governorate, Egypt revealed a decrease in the productivity of acid lime trees, in addition to the appearance of yellowing leaves. It also observed that the soil was compact and cracked. Upon conducting soil analysis, the pH was found to be 8.1, the percentage of bicarbonates was about 2.4 Meq/ liter and the percentage of calcium carbonate was about 10.68 %. Therefore, this study was conducted as an attempt to alleviate this problem and thus increase productivity.

Calcareous soils are extensively distributed in arid and semi-arid environments. It is estimated that these soils cover more than one- third of the world's land surface area. According to the Ministry of Agriculture, calcareous soils cover around 25-30% of Egypt's total land area. The extremely calcareous soils in the North Western Coastal zone (about 3 million feddan) provide excellent opportunities for land reclamation and development to aid in the resolution of the food production of the food production problem, taking into account the available rainfall in the area (**Taalab et al., 2019**). As for agriculture, calcareous soils are considered unproductive. The majority of macro and micronutrients in calcareous soils are not readily available to agricultural plants because they are frequently found in fixed or bound forms. In order to achieve plants growth and development, these nutrients must be liberated from their bound state and made freely available in these soils, it is imperative to apply microbial species that are indigenous to these calcareous soils and possess the potential to liberate these bound nutrients into a form that is readily available (**Borkar et al., 2024**).

Reactive oxygen species (ROS) levels rise as a result of abiotic stress, which also impacts other physiological functions and lowers plant growth and output (**Ashokan et al., 2024**). The over production of reactive oxygen species (ROS) include singlet ( $O_2$ ), superoxide ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $OH^{\bullet}$ ), is a major indicator of such stresses. They induce cellular/ tissue damage when they assault protein, DNA and lipids, etc. leading to irreversible metabolic dysfunctions and ultimately cell death. To overcome these free radicals and neutralize their effects as prooxidants another category of substances termed antioxidants (enzymatic and nonenzymatic) are created either endogenously or obtained from exogenous sources. Superoxide dismutase, catalase, guaiacol peroxidase and glutathione peroxidase are examples of enzymatic antioxidants, non-enzymatic antioxidants include vitamins like vitamin C and E, minerals like Se and Zn, carotenoids, phenolic and flavonoids (**Orabi and Abou-Hussein, 2019**).

Development promoting microorganisms (PGPMs) are microbes that have a favorable association with plants. They can increase the resistance of plant to biotic or abiotic stresses and/ or stimulate plant development. Arbuscular mycorrhizal fungi (AMF) and plant growth promoting bacteria (PGPB) are examples of PGPM (**Bai et al., 2022**). Both direct and indirect mechanisms are possible for PGPMs to work: (i) direct mechanisms include actions that give the plant nutrients or phyto-regulators directly such as phosphorus solubilization, nitrogen fixation, or phytohormone production; (ii) indirect mechanisms increase the plant's resistance to biotic and abiotic stresses by promoting antimicrobial metabolism, triggering induce systemic resistance, or influencing the redox state of the plant (**Kumar et al., 2024**).

Plant growth-promoting bacteria (PGPB) stimulate plant development and soil bioremediation by secreting a variety of metabolites and hormones, fixing nitrogen, and boosting other nutrients bioavailability through mineral's solubilization. Reduced turgor pressure during stress is one of the subtlest physiological mechanisms that permits cells to grow in a stressful environment (**Yan et al., 2020**). PGPB also improves plant remediation ability by encouraging plant growth through the secretion of several types of metabolites and hormones, mineral solubilization, nitrogen fixation, and pathogen protection. PGPB also helps to alleviate a variety of additional biotic and abiotic challenges that plants endure (**Backer et al., 2018**).

Numerous strains of *Bacillus velezensis* species have been shown to inhibit the growth of microbial pathogens including as bacteria, fungus, and nematodes (Mona, et al 2024). Determining the type of biomolecules generated and /or secreted by *B. velezensis* growth under various induced conditions will be our next task. Last but not least, the food and Drug Administration (FAD) has granted many bacillus strains GRAS designation (Generally Regarded as Safe), indicating that they are safe for human consumption. Alongside this discovery, we reported that our *B. velezensis* QA2 strain is susceptible to commonly used antibiotics and may be a safe commercial biotechnological inoculant that may be used as a biocontrol and biofertilizer agent for saline soil (Ismail et al., 2022).

*Cytobacillus firmus* strain can also solubilize phosphours (more than 100 $\mu$  /ml) and hydrolyze phytate, producing IAA. The total growth of barley under salt stress is impacted by the bacterial count in the soil, photosynthesis, and NPK content in the soil and plant, all of which can be increased by using these strains as PGPB biofertilizers. Under salt stress, these strains improve barley's phosphorus management. The nation is increasing its agricultural land, especially saline soils, due to its burgeoning population. The current paradigm of sustainable agriculture emphasizes technique that improve soil fertility naturally and efficiently while protecting the environment (EL-Shaymaa et al., 2024).

More than 200 substances, such as phenols, polyphenols, acetic acid (Velmurugan et al., 2009), ketones, esters, aldehydes, and alcohols (Zhai et al., 2015), can be found in wood vinegar, often referred to as pyroligneous acid. Wood vinegar (WV), a by-product of woody biomass pyrolysis, is increasing being used in agriculture as a sustainable biostimulant, however its effects on plant stress resistance and underlying mechanisms are yet unknown. According to recent research, WV may function through a mechanism based on eustress, which is characterized as a regulated, mild stress that triggers adaptive physiological reactions and improves plant performance without inflicting structural or metabolic harm (Desideri et al.2026). Because of their low pH and high organic content, these chemicals cannot be disposed of without treatment (Fagernas et al., 2012), and must be diluted or neutralized. Depending on its concentration, wood vinegar has strong antibacterial activity against a variety of microbes (Ma et al., 2011; Yang et al., 2016) and can increase microbial activity in soils (Steiner et al., 2008).

The objective of this study was to investigate two specie's of plant growth-promoting bacteria (*Bacillus velezensis* or *Cytobacillus firmus*) and wood vinegar in addition as a soil treatment as well as enhancement lime yield and fruit quality under these soil conditions.

## 2. Materials and Methods

The present study was carried out during two successive seasons 2022 and 2023 in a private citrus orchard in El- azyma city, El – Beheira governorate, Egypt, 7 years- old seed- grown acid lime trees (*Citrus aurantifolia* Swingle) in calcareous soil at 6 $\times$ 7 m under drip irrigation system using Nile River water, were used to investigate the influence of different soil treatments on the yield and fruit quality of acid lime fruits.

The initial soil sample was collected from the root zone (rhizosphere) and analyzed for some physical and chemical characteristics (Table 1).

**Table (1). Some physical and chemical properties of the studied soil**

pH (1:2.5)	EC (mmhos/cm)	Cations and anions (Meq/ liter)								Physical properties (%)			Textural class
		Na <sup>+</sup>	K <sup>+</sup>	Ca <sup>++</sup>	Mg <sup>+</sup>	CO <sub>3</sub> <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	Cl <sup>-</sup>	SO <sub>4</sub> <sup>-</sup>	Sand	Slit	Clay	
8.1	0.66	4.1	0.5	1.4	0.6	-	2.4	3.2	1	78	10	12	Sandy loam

Soil analysis also showed that the percentage of organic matter was 0.47%, the percentage of calcium carbonate was 10.68 %, and the available phosphorus was 18 Meq/liter.

Thirty-six trees (three replicates with three trees at each replicate) were subjected to four investigated treatments as follows:

1. Control (Uniform fertilizer program).
2. *Bacillus velezensis* at the rate of 100 ml/ tree ( $7.5 \times 10^7$  cfu/ml).
3. *Cytobacillus firmus* at the rate of 100 ml/ tree ( $6.2 \times 10^9$  cfu/ml).
4. Wood vinegar at the rate of 250 ml/ 3750 ml water /tree (at a 1:15 dilution).

For each season, all treatments were added to the soil three times during the second week of March (full bloom stage), May (fruitlet cell division), and July (fruit cell expansion). One way randomized block design was used, each treatment was replicated three times with three trees for each replicate, and thus the total number of trees in this experiment was 36 trees (4 treatments x 3 replicate x 3 trees in each replicate).

### 2.1. Microbial strains

*Bacillus velezensis*: an aerobic, gram-positive, w=endospore-forming bacterium that promotes plant growth, was identified from strawberry (*Fragria chiloensis var ananassa*) no. OQ073573 by the soil water and environmental research institute (SWERI).

*Cytobacillus firmus*: the OR341132 strain of *Cytobacillus firmus* was isolated from mangrove forest in Ras Muhammad Nature Reserve located 12 km from the city of Sharm El Sheikh at the southern extremity of the Sinai by soil water and environmental research institute (SWERI). Gram-positive aerobic chemoheterotrophic bacteria that can withstand a salt concentration of up to 10%, fix nitrogen by growing on nitrogen-free media with exceptional growth, and hydrolyze phytate and IAA in addition to solubilizing phosphorus at levels greater than  $100\mu\text{/ ml}$  of phosphate, ad demonstrated both qualitatively and quantitatively.

### 2.2. Wood vinegar

The wood vinegar used in our experiment is a commercial compound and was analyzed according to soil, water and environmental unit at soil, water and environmental research institute as shown in the following Table (2).

**Table (2). Wood vinegar analysis**

Minerals	Concentrations
N	0.90 %
NH <sub>4</sub>	0.79%
NO <sub>3</sub>	0.08%
K <sub>2</sub> O	*<1%
P	100 mg/l.
MgO	50.4 mg/l.
CaO	302
Fe	0.13 mg/l.
Mn	12.4 mg/l.
Zn	5.8 mg/l.
Cu	2.9 mg/l.
pH	4.12
EC (ds/m)	0.095
Total amino acids	2.85%

### 2.3. The following parameters were investigated:

#### 2.3.1. Fruit yield

Each tree's fruits were collected to determine yield (kg/ tree) during harvest time, which in these experimental condition is the third week of August.

#### 2.3.2. Fruit quality

Fruit physical characters; Average fruit weight (g) and fruit juice percent (%) as well as fruit chemical character; TSS %, acidity % (as mg citric acid/100 cm juice), TSS/ acid ratio and vitamin C (ascorbic acid as mg/100ml juice) were determined according to (A.O.A.C 1995).

#### 2.3.3. Plant pigments

Fresh leaves from the two experimental seasons were gathered in the second week of August (one month after the last treatment addition date), extracted using dimethyl formamide (D.M.F) solution [HCON(CH<sub>3</sub>)<sub>2</sub>], and left overnight at a cool temperature (50C). A spectrophotometer was used to measure chlorophyll a, b, and total chlorophyll (Nornai., 1982).

#### 2.3.4. Plant defense system

In the second week of August at the two experimental seasons; Proline concentration was determined by spectrophotometry in dry leaves as described in (Bates *et al.*, 1973). Total phenols were determined in fresh leaves according to (Zieslin and Ben-Zaken., 1993). Leaves ascorbic acid (ASA) was determined in fresh leaves according to (Oser., 1979). Leaves peroxidase (POD) concentration was determined according to (Maehly, 1954).

#### 2.3.5. Minerals concentration

N, P and K concentrations were determined in dried leaves at the end of the two experimental seasons. Total N% was determined by semi-micro Kjeldahl method described by (Plummer., 1971). Phosphorus was estimated colorimetrically as described by (King., 1951). Potassium concentration was determined by using the flame photometer.

#### 2.3.6. Soil Analysis

Soil samples from all treatments were taken during the third week of August at the end of the two experimental seasons in order to measure the soil-pH (which was recorded in 1:2.5; soil: water suspension) using a pH-meter as described by (Page *et al.*, 1982). Soil microbial activity represented by measuring CO<sub>2</sub> evolution according to (Pramer and Schmidt., 1964). The dehydrogenase activity in soil was determined according to (Thalman., 1967). Soil IAA was determined according to (Sarwar *et al.*, 1992). Also, available N, P and K (mg/ kg soil) were determinate according to (Cottenie *et al.*, 1982).

### 2.4. Statistical analysis

The experiment employed a one-way randomized block design. According to (Snedecor and Cochran., 1980), the obtained data was statistically evaluated to ascertain the analysis of variance and the differences between means. Using MSTAT-C software, multiple comparisons of means were carried out in accordance with Duncan's multiple test range (Freed and Scott., 1986).

## 3. Results and discussion

### 3.1. Fruit yield

It is clear from data presented in Table (3) that, applied treatments led to significant increment in yield (kg/ tree) as compared with control at the two experimental seasons. The positive effect of the treatments on the fruit yield is due to their ability to reduce the stress on acid lime trees grown in calcareous soils. The highest values of fruit yield per tree were recorded by wood vinegar treatment at the two successive seasons as compared with control or other treatments.

In this respect, in general, wood vinegar can increase microbial activity and crop yield. However, the majority of earlier research independently verified the impact of wood vinegar on plants and soil microorganisms (Jeong *et al.*, 2015). Moreover, applying wood vinegar greatly increased fruit yield, which may be connected to its function as a biostimulant rich in organic acids, phenolic compounds, and growth-promoting substances can enhance root development, nutrient availability, and soil microbial activity, which will raise plant biomass and production. According to earlier research, using wood vinegar raised crop output by an average of 21-31% in a variety of environmental settings (Jens and Iva, 2025), and the results did not introduce the coupling relationship, particularly the regulation of rhizosphere microorganisms through wood vinegar leading to crop growth.

**Table (3). Effect of treatments on acid lime yield and some fruit quality characters**

Treatments	Yield (kg/ tree)	Fruit weight (g)	Fruit juice (%)	T.S.S/ acid (%)	Acidity (%)	TSS (%)	V.C (mg/100 ml juice)
	First season						
Control	33.00 d	29.32 d	25.60 c	0.95 d	7.40 ab	7.00 c	50.75 a
<i>Bacillus velezensis</i>	47.55 c	32.63 c	31.95 b	0.98 c	7.66 a	7.50 b	45.63 a
<i>Cytobacillus firmus</i>	50.48 b	36.34 a	36.80 a	1.03 b	7.29 b	7.50 b	48.00 a
Wood vinegar	54.07 a	33.39 b	35.45 a	1.05 a	7.33 b	7.69 a	48.25 a
Second season							
Control	35.08 d	29.16 c	25.57 c	7.55 ab	0.94 b	7.13 b	48.00 a
<i>Bacillus velezensis</i>	49.00 c	35.48 b	32.87 b	8.14 a	1.047 a	8.49 a	50.75 a
<i>Cytobacillus firmus</i>	53.53 b	38.39 a	37.40 a	7.66 ab	1.02 ab	7.78 ab	47.00 a
Wood vinegar	60.11 a	35.17 b	33.54 b	6.99 b	1.08 a	7.53 b	49.83 a

Means with the same letter are not significantly different at 0.05 level of probability.

Thus, the effects of wood vinegar composition on soil microorganisms' response and cherry radish growth promotion were investigated, and its uses were further identified (Gu *et al.*, 2024). As for the effect of plant promoting bacteria in increasing the yield as compared with control (Table 3), it may be due to the fact they induce the production of certain hormones such as auxin and gibberellins which in turn increases the yield.

In this respect, interactions between roots and soil microbiota have a significant impact on plant nutrition. Through the synthesis of plant hormones (auxin and gibberellins), nitrogen fixation, saturation of inorganic phosphate, and mineralization of organic phosphate, soil bacteria help plants thrive (Richardson *et al.*, 2009). In general, auxin is carried to the roots, where it stimulates the roots, longer and more branched roots also take more nutrients, which are then carried to the sink, increasing the total yield (Wang *et al.*, 2005). By regulating senescence (Ellis *et al.*, 2005), fruit production (De Jong *et al.*, 2009), and abiotic stress resistance (Wang *et al.*, 2010), auxin influences plant development. In addition to delaying senescence and aiding in fruit growth and development, auxin also has a slight impact on the start of flowers and the development of reproductive organs (Asahira *et al.*, 1967). Also, gibberellic acid has been suggested to enhance many crop cycle and yield- related activities in citrus species (Garmendia *et al.*, 2019). Also, stress, whether abiotic or biotic, has a deleterious impact on plant growth, development, and yield (Ashraf *et al.*, 2019). The addition of biofertilizers to soil is crucial for increasing soil fertility, yield-attributing traits, and ultimately, yield. Agriculture yields are moderately increased by biofertilizers because they improve soil and plant health and increase the availability of nutrients to agricultural plants (Kumar and Sarkar, 2019).

### 3.2. Fruit quality

#### 3.2.1. Physical characters

The highest significant fruit weight and fruit juice percent values were recorded for *Cytobacillus firmus* treatment as compared with control (Table 3). In this respect, in an integrated fertilization system, the application of organic and biofertilizers is essential for enhancing soil properties and sustaining crop productivity for horticultural crops (Pathak *et al.*, 2017). Because of this, organic and biofertilizers release nitrogen gradually, which causes the fruit to develop larger throughout the course of its growth period. The fruit pulp's water content rises as a result of the biofertilizer's increased peel water content and fruit easier absorption of potassium (Debnath *et al.*, 2019). The increment effect of both fruit weight and fruit juice percent by *Cytobacillus firmus* addition may be due to this treatment increasing the auxin production, which in turn increases fruit development. In this respect, in addition to plants, bacteria and fungi can also synthesize IAA (Duca *et al.*, 2014). Auxins are one of the phytohormones that play a key role in a number of essential plant development and stress response activities. Auxins control the following processes; (i) adventitious and lateral root formation; (ii) shoot branching and leaf development; (iii) plant flowering and fruit production; and (iv) resistance to biotic and abiotic stress [50]. Indole -3- acetic acid is the most prevalent and significant auxin found in plants. IAA controls cambial growth cambial growth, vascular development, flower and fruit development, root creation, and cell division and elongation (Duca *et al.*, 2014).

#### 3.2.2. Chemical characters

The data in Table (3) showed that, the highest values of fruit acidity were recorded by *Bacillus velezensis* treatment at the two experimental seasons as compared with control. In this respect, it's interesting to note that we observed that *Bacillus velezensis* QA2 has exceptional biocontrol effectiveness against common bacterial and fungal diseases that affect both humans and plants, in addition to the PGPB (plant growth prompting bacteria) features. The biochemical, physiological, and secondary metabolites characteristics of *B. velezensis* QA2 are linked to its antagonistic qualities. The current investigation creates a fresh opportunity to examine the fundamental processes by which *B. velezensis* QA2 reduces salt stress (Ismail, 2022).

Regarding to total soluble solids (TSS), data illustrated that, in the two experimental seasons all treatments increased the TSS% as compared with control. While, the highest values of TSS/ acid ratio were detected by wood vinegar treatment with compared with control. Concerning to vitamin C, the data revealed that no detected trend was observed by all treatments.

In this respect, since organic and biofertilizers can enhance soil health, boost yields, and improve fruit quality while preserving environmental sustainability, they are essential to sustainable horticultural crop production systems (Hazarika and Aheibam, 2019). Also, wood vinegar is a natural product that improves soil quality, improves fruit quality and sugar content and promotes crop resistance (Mmojieje and Hornung 2015). The number of fruits, their fresh and dry weights, and their overall dry weight all increased when wood vinegar was added. Furthermore, it successfully raised the fruit's total soluble solutes (Abdel Aziz *et al.*, 2023).

#### 3.2.3. Plant pigments

Data represented in Table (4) demonstrated that, in the two experimental seasons the highest values of chlorophyll b and total chlorophyll concentrations were detected by *Cytobacillus firmus* followed by wood vinegar as compared with control with some exceptions. In this respect, the amount of chlorophyll has a significant impact on the rate of photosynthetic activity and the creation of dray matter (Fathi and Zeidali, 2021). Also, nitrogen is important in photosynthesis and is a crucial part of chlorophyll. Gaseous nitrogen ( $N_2$ ) was transformed into ammonia ( $NH_3$ ) by nitrogen-fixing PGPB, making it accessible to host plants (Kaleh *et al.*, 2022). As an essential component of chlorophyll, amino acids, and nucleic acids, nitrogen is similarly important to plants. It has been demonstrated in

the past that interactions with *Bacillus* species can increase the content of nitrogen in non-leguminous plants (Thatoi *et al.*, 2020). Moreover, increasing plant growth, photosynthesis rate, transpiration, oxidative defense capacity, and photosynthetic pigments, ascorbic acid can increase resistance to abiotic stressors (Akram *et al.*, 2017). Bacterial IAA production stimulates photosynthesis, biomass, and plant growth (Duca and Glick, 2020).

**Table (4) Effect of treatment on leaves plant pigments, proline and total phenols concentration.**

Treatments	Chl. a (mg/g.f.w)	Chl. b (mg/g.f.w)	Total chl. (mg/g.f.w)	Proline (ppm)	Total phenols (mg/g.f.w)
	First season				
Control	1.030 b	0.4467 c	1.477 c	1.19 d	1.86 a
<i>Bacillus velezensis</i>	1.200 a	0.5500 b	1.750 b	1.83 b	1.14 b
<i>Cytobacillus firmus</i>	1.200 a	0.7100 a	1.913 a	2.09 a	0.99 c
Wood vinegar	1.200 a	0.5767 b	1.773 b	1.44 c	1.02 c
Second season					
Control	1.19 b	0.38 c	1.57 c	1.05 d	1.84 a
<i>Bacillus velezensis</i>	1.20 a	0.54 b	1.73 b	1.93 b	0.97 b
<i>Cytobacillus firmus</i>	1.20 a	0.69 a	1.88 a	2.24 a	0.98 b
Wood vinegar	1.19 b	0.65 a	1.83 a	1.66 c	1.03 b

Means with the same letter are not significantly different at 0.05 level of probability.

Concerning to the effect of wood vinegar, it is well documented that, in potatoes, wood vinegar ester compounds have been shown to improve the formation of chlorophyll, increase photosynthesis, produce more sugars and amino acids, and increase plant resilience to pests and diseases (Chuaboon *et al.*, 2016). To put it briefly, applying a 200 –fold diluted wood vinegar greatly increased the cherry radish's photosynthetic rate, transpiration, root growth, and chlorophyll content, improving nutrient absorption and efficient use of intercellular CO<sub>2</sub> (Gu *et al.*, 2024). By improving the plant's capacity to absorb light energy, an increase in chlorophyll content may successfully support photosynthesis, resulting in better growth, increased biomass production, and enhances plant health overall (Li *et al.*, 2020).

### 3.4. Plant defense system

#### 3.4.1. Proline

Data represented in Table (4) demonstrated that, the highest values of proline concentration were recorded by *Cytobacillus firmus* followed by *Bacillus velezensis* as compared with control for both seasons of the study. In this respect, numerous phytohormones found in biofertilizers that promote citrus growth by boosting photosynthesis, improving nutrient uptake, regulating plant cell division and size, and boosting proline biosynthesis. Numerous studies have discovered that plants inoculated with these biofertilizers release a variety of phytohormones, such as cytokinins and indole acetic acid. Therefore, biofertilizers are essential for increasing citrus plant and fruit quality and output (Zahgloul *et al.*, 2015). The activation of osmoprotective system, which enable plants to withstand abiotic stress conditions such high soil alkalinity, is shown by the rise in proline concentration seen in treated plants. It is well known that proline accumulation serves as a defense mechanism that improves antioxidant capacity, preserves cellular osmotic balance, and stabilizes proteins under stress (Vives-Peris *et al.*, 2017).

#### 3.4.2. Total phenols

It is generally clear from the results in Table (4) that, throughout the two experimental seasons the lowest values of total phenols concentrations were recorded by *Cytobacillus firmus* as compared with control. This is probably because this treatment reduced the effect of stress on acid lime trees grown in calcareous soils.

In this respect, plants produce reactive oxygen species (ROS) in almost all stress settings, and in response, they make phenolic acids, polyphenols, flavonoids, anthocyanins, and other chemicals with properties comparable to counter ROS, such as H<sub>2</sub>O<sub>2</sub> (Salunke and Koche, 2023). Plants develop the ability to produce distinct phenolic compounds in response to specific abiotic or biotic stressors over time through adaptive evolutionary processes. Plants have evolved a number of arrays to synthesis various phenolics to protect against environmental stressors (Caputi *et al.*, 2012). Salinity stress typically causes plants to produce reactive oxygen species (ROS), which leads to oxidative stress. To offset this oxidative damage plants, create antioxidative metabolites such as polyphenols, flavonoids, anthocyanins, pro-anthocyanidins phenolic acids, and phenolic terpenes which quench the singlet oxygen, neutralize or absorb free radicals and destroy peroxides (Oh *et al.*, 2009).

### 3.4.3. Ascorbic acid

Concerning to leaves ascorbic acid concentration, the data in Table (5) revealed that, at the two experimental seasons all treatments increased the ascorbic acid concentration, the highest values were obtained by *Cytobacillus firmus* followed by *Bacillus velezensis* as compared with control.

**Table (5). Effect of treatments on leaves ascorbic acid, peroxidase, N, P and K concentrations**

Treatments	Ascorbic acid (mg/g. f. w.)	Peroxidase (U/mg)	N%	P%	K%
	First season				
Control	13.90 b	0.68 b	1.70 c	0.12 c	1.01 d
<i>Bacillus velezensis</i>	26.43 a	0.81 b	2.25 ab	0.23 ab	1.51 b
<i>Cytobacillus firmus</i>	27.18 a	0.69 b	2.18 b	0.18 b	1.38 c
Wood vinegar	24.98 a	1.09 a	2.27 a	0.25 a	1.68 a
Second season					
Control	12.36 d	0.50 c	1.76 c	0.13 c	0.92 d
<i>Bacillus velezensis</i>	25.85 a	0.87 b	2.26 a	0.24 ab	1.41 c
<i>Cytobacillus firmus</i>	27.32 a	0.86 b	2.15 b	0.19 b	1.56 b
Wood vinegar	23.94 c	1.70 a	2.32 a	0.27 a	1.91 a

Means with the same letter are not significantly different at 0.05 level of probability.

In this respect, ascorbic acid (ASA), the antioxidant vitamin C, is an essential substrate for the detoxification of reactive oxygen species (Qian *et al.*, 2014). Reactive oxygen species (ROS) over-production in stressed plants is a regular occurrence. Plants typically combat this issue by producing ROS-neutralizing compounds, such as enzymatic and non- enzymatic antioxidants. In this regard, ascorbic acid (ASA) is one of the ubiquitous non-enzymatic antioxidants with significant potential for both scavenging reactive oxygen species (ROS) and modifying several essential plant processes under both stress and non-stress situations (Akram *et al.*, 2017). It is well known that ascorbic acid shields cells and organelles from the enemies of reactive oxygen species (ROS), which build up excessively as a result of oxidative damage brought on by stress (Naz *et al.*, 2016). It regulates cell growth and division, functions as a cofactor for several enzymes, modifies plant perception, and is involved in hormone biosynthesis, photosynthesis, and antioxidant renewal (Lisko *et al.*, 2014).

### 3.4.4. Peroxidase (POD)

Data represented in Table (5) showed that, the peroxidase concentration recorded the highest values by wood vinegar treatment in both seasons as compared with control. In this respect, in response to certain abiotic stress situations, plants will exhibit an increase in ascorbate peroxidase activity (Abd El-motty and Orabi, 2013). Additionally, there is proof that applying wood vinegar improves the microbiological conditions in the soil (Koç *et al.*, 2019) increasing the availability of

nutrients (Jeong *et al.*, 2015) and soil enzyme activity (Lashari *et al.*, 2013). By producing more antioxidants including catalase, peroxidase, superoxide dismutase, flavonoids, and phenolic substances, beneficial bacteria may shield the plant from rising ROS levels (Abdelaal *et al.*, 2021).

The data for proline, peroxidase, ascorbic acid, and phenols (Tables 4, 5) make it evident that, with a few exceptions, acid lime trees treated with bacterial inoculation (*Cytobacillus firmus*) resulted in higher concentrations of proline and ascorbic acid when compared to control or other treatments in the two experimental seasons. Furthermore, both plant growth prompting bacteria ranks second in peroxidase enzyme concentration after wood vinegar in the second season compared with control. All of these compounds collectively increased the plant's defense mechanism to withstand stress under calcareous soil conditions, even while phenols showed the reverse trend. On the other hand, when a plant is exposed to stress, it begins to produce proline internally, which removes free radicals from the cell and increases the activity of antioxidant enzymes like peroxidase, which reduces oxidative damage and eliminates reactive oxygen species (ROS). Consequently, this helps the cells recover from the damage caused by stress and maintains vital activities.

In this respect, through a rise in antioxidant molecules as catalase, peroxidase, flavonoids, and phenolic compounds, beneficial bacteria may shield the plant from rising ROS levels (Abdelaal *et al.*, 2021). Ascorbic acid- regulation of antioxidant defense metabolism in many plants cultivated under stress circumstances, such as canola under salinity, has been reported in a number of studies (Bybordi, 2012). This has mostly been attributed to ASA's role in the activation of various antioxidant enzymes are heme-containing antioxidative enzymes that are known to convert H<sub>2</sub>O<sub>2</sub> to water and molecular oxygen utilizing ASA as an electron source, among other antioxidative enzymes known to be involved in the antioxidant defense system (Van Doorn and Ketsa, 2014). Plants ability to adapt to harsh environmental circumstances is enhanced by the production of phenolic compounds during biotic and abiotic stress. Understanding the molecular mechanism controlling the creation and accumulation of different phenolic compounds under distinct stress circumstances is therefore essential. The regulation of transcription factors involved for the molecular and genetic levels (Salunke and Koche, 2023). Also, Non-enzymatic defenses include substances of intrinsic antioxidant capabilities, water soluble such as ascorbate (vitamin C), glutathione, phenolic compounds and flavonoids. Superoxide dismutase (SOD), catalase, guaiacol peroxidase and glutathione peroxidase are the primary enzymatic defenses or scavengers that protect cells by directly scavenging superoxidem converting them to less reactive species (Orabi and Abou-Hussein, 2019).

### 3.5. Leaves minerals concentration

As regard to leaves minerals concentrations, results indicated that, in the two experimental seasons the highest values of nitrogen, phosphorus and potassium concentrations were obtained by wood vinegar application followed by *Bacillus velezensis* as compared with control (Table 5). In this respect, alcohols, esters, amines, pyridines, and some elements like potassium, phosphorus, calcium, manganese, and iron were found in wood vinegar, which was used as a soil remediation agent (Lu *et al.*, 2020). Wood vinegar can improve soil fertility by increasing nutrient elements (such as N, P and K) of citrus rootstocks (Mirsoleimani *et al.*, 2023). Also, wood vinegar appears to increase cell growth and functions as a stimulant for the growth of certain microorganisms as well as enzyme activation. The latter are crucial for a variety of physiological and metabolic activities, nutrients uptake, as well as cell proliferation, although the mechanisms involved are poorly understood (Abdel Aziz *et al.*, 2023).

Concerning the *Bacillus velezensis* effect, soil microorganisms are important in decomposing organic materials and recycling their nutrients for uptake by plants (Higa and Wididana, 1991). Bacterial IAA production, by increasing the quantity and length of adventitious and primary roots, IAA production promotes root proliferation, which improves water and nutrient uptake and mitigates the impacts of salinity and drought (Etesami and Maheshwari, 2018).

### 3.6. Soil Analysis

#### 3.6.1. Soil pH

Concerning to pH the data in Fig (1) revealed that, the lowest values of soil pH were recorded by wood vinegar treatment at the two experimental seasons as compared with control. The reason why wood vinegar able to lower the pH is due to the fact that one of its slandered properties is that its pH is around 3.

In this respect, the Japan Pyroligneous Liquor Association, an industrial association for the pyroligneous liquor sector, has established seven criteria to standardize vinegar quality. One of these criterion is the vinegar's pH, which has to be around 3 (Wada, 1997). Additionally, wood vinegar serves as a fungicide targeting soil-borne and foliar phytopathogenic fungi. Beyond its pesticidal properties, it contributes to soil health by buffering pH and stimulating beneficial microbial activity (Shetty *et al.*, 2025).

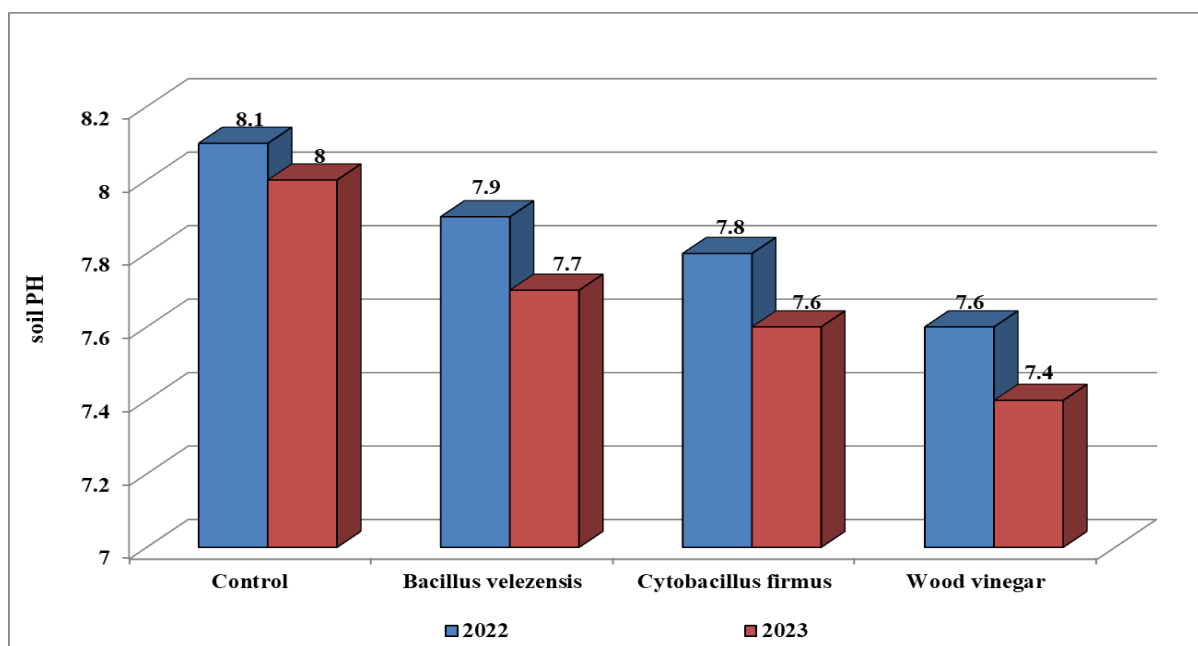


Fig (1): Effect of treatments on soil pH at the two successive seasons.

#### 3.6.2. CO<sub>2</sub> evolution

It is clear from the obtained data in Table 6 that, in the two successive seasons using both plant growth prompting bacteria increased significantly the soil CO<sub>2</sub> concentration as compared with control treatment. The high soil CO<sub>2</sub> values using plant growth promoting bacteria, compared to the control, may be due to the respiration produced by these microbes that reveals the actual biological activity of bacteria within the soil. In this respect, emissions of soil carbon dioxide (CO<sub>2</sub>) have some feedback effects on the dynamic of the soil carbon pool, which is important for managing agroecosystems and assessing climate change (Zhang *et al.*, 2025).

#### 3.6.3. Dehydrogenase activity

As regarding dehydrogenase activity, data in Table (6) demonstrated that, highest values were recorded by *Cytobacillus firmus* followed by *Bacillus velezensis* as compared with control at the two experimental seasons. In this respect, dehydrogenase activity is a vital component of soil microbial (Włodarczyk *et al.*, 2002). Applying biofertilizer may increase the activity of soil enzymes. According to a similar investigation, the rise in enzymatic activities may be explained by variations in rhizosphere bacterial activity (Zhou, 2011).

Since dehydrogenase earning hydrogen from organic matter and releases energy during microbial respiration, which increases CO<sub>2</sub> production, it is evident from the results that there is a positive correlation between dehydrogenase activity and carbon dioxide emission in soil. Consequently, a rise in either metric indicates biological activity and vitality in the soil.

### 3.6.4. Soil IAA

Data represented in Table (6) revealed that, in the two experimental seasons the highest values of soil IAA were detected by *Cytobacillus firmus* followed by *Bacillus velezensis* as compared with control, with some exceptions.

In this respect, under in vitro conditions, the *Cytobacillus firmus* strain was discovered to be capable of producing the phytohormones IAA, ammonia, HCN, and siderophore, as well as phosphate solubilization. This suggests that *Cytobacillus firmus* can be utilized as bio-inoculants, bio-fertilizers, and biocontrol agents instead of chemical fertilizers to improve crop growth and productivity without causing environmental pollution or health risks. Testing the potential of plant growth boosters in pots or in situ is crucial for future formulation as a bio-inoculant for farmers' usage (Ganvir *et al.*, 2023). Also, their superiority point stems from their capacity to fix nitrogen, solubilize phosphorus, hydrolyze phytate, and produce IAA (EL-Shaymaa *et al.*, 2024).

**Table (6). Effect of treatments on soil analysis; soil CO<sub>2</sub>, dehydrogenase and IAA**

Treatments	Soil CO <sub>2</sub> (mg/g. day)	Soil dehydrogenase (mg/g. day)	Soil IAA (mg/ Kg)	Soil CO <sub>2</sub> (mg/g. day)	Soil dehydrogenase (mg/g. day)	Soil IAA (mg/ Kg)
	First season			Second season		
Control	3.67 b	0.36 c	35.50 d	2.57 c	0.38 c	34.76 c
<i>Bacillus velezensis</i>	5.52 a	0.75 b	79.22 b	4.44 a	0.77 b	72.08 a
<i>Cytobacillus firmus</i>	5.74 a	0.92 a	91.86 a	4.68 a	1.08 a	73.52 a
Wood vinegar	4.13 b	0.34 c	45.76 c	3.36 b	0.38 c	51.26 b

Means with the same letter are not significantly different at 0.05 level of probability.

### 3.6.5. Available nutrients content

It is clear from the data presented in Figure (2) that, in the two successive seasons there are a decreases in soil nutrients content (N, P and K) were detected by all treatments as compared with control. The lowest values were observed with wood vinegar treatment compared with control and the other treatments. This noticeable deficiency in soil nutrient content compared with control may be due to the availability of these nutrients and the ease of their absorption by lime trees treated with different treatments.

In this respect, (Lu *et al.*, 2019) found that applying wood vinegar at low concentrations (0.33-0.50 ml/L) increased wheat fresh root biomass and root vitality. Additionally, the application of wood vinegar increased soil organic matter because it stimulated populations of beneficial microbes and a more labile C pool, which improved plant uptake of nutrients (Wang *et al.*, 2022). A 500-fold diluted wood vinegar treatment of the soil ecological activity and the number of bacteria (including Gram-negative, anaerobic, and aerobic bacteria) by 73.39% ( $p < 0.05$ ) (Rui *et al.*, 2014).

Concerning to PGPB impact, gaseous nitrogen (N<sub>2</sub>) was transformed into ammonia (NH<sub>3</sub>) nitrogen – fixing PGPB, making it accessible to host plants (Kaleh *et al.*, 2022). As an essential component of chlorophyll, amino acids, and nucleic acids, nitrogen is similarly important to plants. It has been demonstrated in the past that interactions with *Bacillus* species can increase the content of nitrogen in non- leguminous plants (Thatoi *et al.*, 2020). Also, in the natural world, plants need the help of the microbial community to mineralize and dissolve both forms of phosphate. In the meantime, nonspecific acid phosphatases, alkaline phosphatases, and phytases that are spontaneously produced by bacteria are responsible for the solubilization of organic phosphate through mineralization (Lindang *et al.*, 2021).

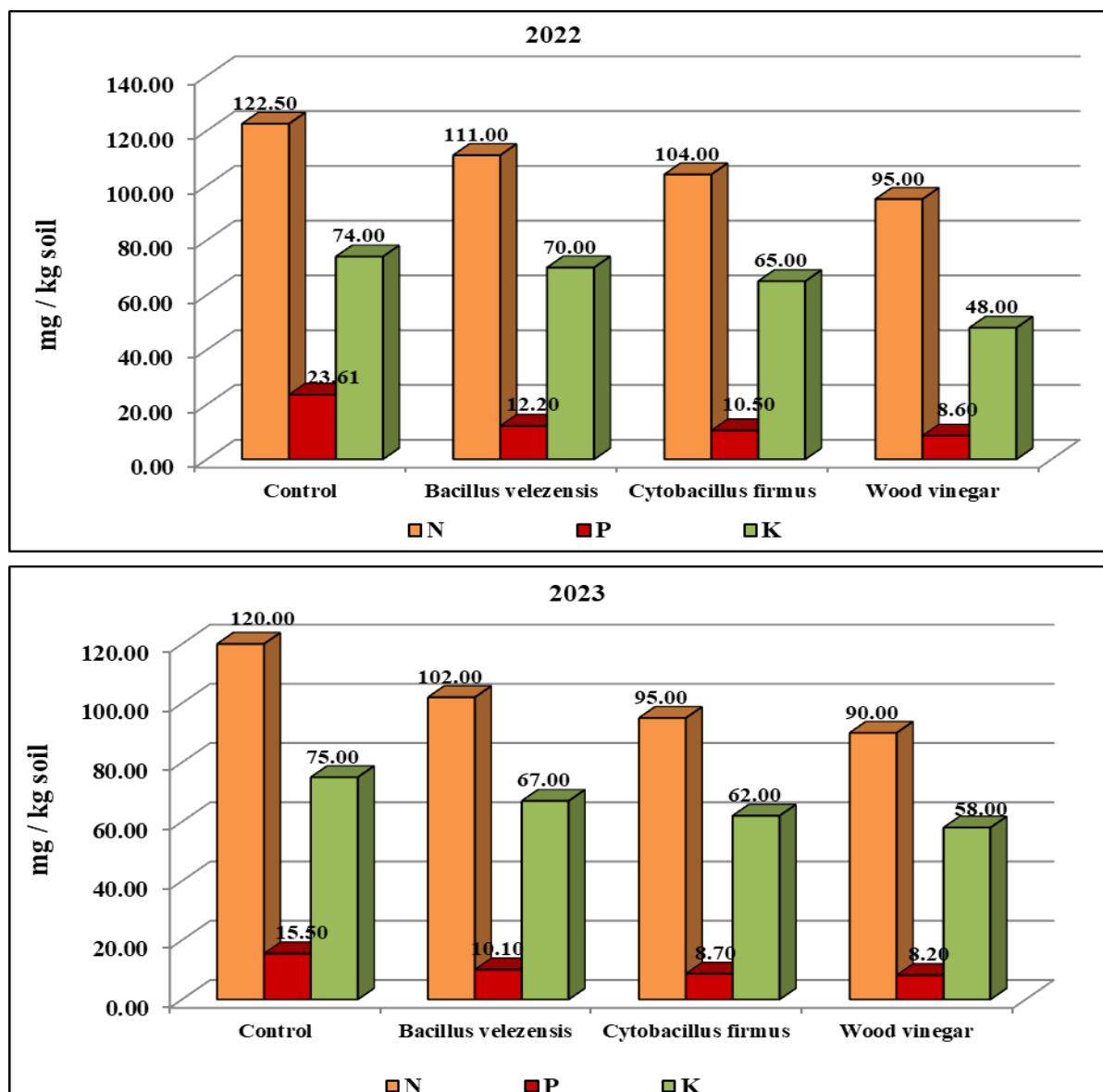


Fig (2). The effect of treatments on available N, K, P in soil at the two successive seasons

#### 4. Conclusion

We can conclude that, wood vinegar showed the highest values of fruit yield, peroxidase and leaves minerals concentration. While, *Cytobacillus firmus* treatment resulted in increasing acid lime fruit weight, fruit juice percent, chlorophyll b and total chlorophyll, total proline, ascorbic acid concentrations as well as decreasing the total phenols concentration as compared with control. Moreover, these treatments recorded the highest values of all soil analysis (soil CO<sub>2</sub>, dehydrogenase and IAA). Although, the wood vinegar treatment recorded the highest values of fruit yield during the two experimental seasons, while the *Cytobacillus firmus* treatment came in second in terms of yield quantity, we recommend these treatment because of their advantages, as they were isolated from calcareous soils and can lives under these conditions, facilitates the absorption of nitrogen and some other elements by the plant, also, because of their effect on increasing the plant's defense system by increasing the concentrations of both proline, ascorbic acid and decreasing the concentration of phenols substance, in addition of its effect on auxin production and increasing the activity of the dehydrogenase enzyme. Also, it is possible to conduct more studies on the possibility of mixing both wood vinegar and *Cytobacillus firmus*, given their advantages in treating calcareous soils by facilitating elements absorption and increasing the activity of some enzymes in the soil, and the yield and fruit quality characteristics of acid lime.

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