

Optimization of indigenous rhizobacteria in tomato and potato rhizosphere as a source of cytokinins to reduce drought stress

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ABSTRACT

The use of organic materials is currently a concern in developing plant commodities. Important organic materials such as fertilizers, pesticides, and phytohormones. The phytohormone cytokinin is one of the important hormones for plants because cytokinin can help growth and make plants tolerant to abiotic stress. One that can produce cytokinin is rhizobacteria. This study explores the potential of rhizobacteria as producers of cytokinin phytohormone against plant abiotic stress. Rhizobacteria from tomato and potato plants were isolated using standard m63 media and tested for metabolomics using GC-MS. The results showed that these rhizobacteria can produce various cytokinin derivative metabolites such as Trans Zeatin, cis Zeatin, and Kinetin. These metabolites function to increase plant tolerance to abiotic stress. This study uses a bioinformatic approach to GC-MS results to map the characteristics of metabolites that are potential for plants to deal with abiotic stress so that they can become a development strategy to increase productivity under abiotic stress conditions. The results showed that the three isolated bacteria produced higher concentration of kinetin 7N glucosinolate form compared to other cytokinin derivatives. The concentrations were 2.79%, 2.08% and 2.14% in isolate IRT1 10^{-3} , IRT3 10^{-2} and IRT3 10^{-4} , respectively.

Keywords: rhizobacteria, cytokinins, trans zeatin, cis zeatin, drought stress.

INTRODUCTION

The use of organic materials has recently been increasingly studied and observed. Its important role in increasing plant growth and tolerance to environmental stress, both biotic and abiotic. Drought stress is one of the major problems in the world. This condition causes plants to not grow optimally (Zargar et al., 2017). Drought can also significantly inhibit plant growth caused by decreased water content in plant tissue, decreased turgor pressure, and decreased photosynthesis (Salehi-Lisar et al., 2016). Organic materials such as microorganisms have been widely used in agriculture, especially in increasing plant growth and ameliorating stress response (Drobek et al., 2019). Rhizobacteria play a role in increasing plant growth by providing essential nutrients needed by plants such as nitrogen, phosphorus and other

micronutrients (Khoshru et al., 2020). Several rhizobacteria families such as *bacillus* and *pseudomonas* are clearly symbiotic with plants in providing the required nutrients (Pii et al., 2015). On the other hand, the role of rhizobacteria is in providing important phytohormones for plants, such as auxin, gibberellin and cytokinin. In this study, the isolated rhizobacteria focused on their role in producing cytokinin (Patel and Saraf, 2017).

Cytokinin is one of the growth hormones that is very important in the process of cell differentiation and the growth of shoots and roots (Mok, 2019a). It also plays a role in increasing plant defense against environmental stress in the form of abiotic stress such as drought, flood, high temperature, salinity, and heavy metal (Li et al., 2022). Cytokinin compounds such as trans zeatin, cis zeatin and kinetin are produced by several rhizobacteria with different concentrations (Nieto and

Frankenberger Jr, 2017). These compounds play an active role in mitigating abiotic stress through the process of closing and opening stomata, increasing the process of photosynthesis and plant growth (Bielach et al., 2017a). The application of cytokinin compounds to plants is expected to contribute to increasing yields and tolerance to abiotic stress (Godoy et al., 2021).

Tomatoes (*Solanum lycopersicum* L.) and potatoes (*Solanum tuberosum* L.) are two important agricultural commodities that provide great benefits to the world's population (Ahmadu et al., 2021). These commodities are among the most widely cultivated plants that are susceptible to drought conditions (Cui et al., 2019). The use of rhizobacteria isolated from tomato and potato roots is expected to produce important phytohormones for plant growth and increase drought stress tolerance. This study aims to evaluate the role of cytokinin compounds produced by indigenous rhizobacteria isolated from the tomatoes and potatoes.

MATERIAL AND METHODS

Bacterial isolation and characterization

Rhizobacteria were isolated from the rhizosphere of tomato and potato plants at the UMM Malang research field and in farmers' fields in Sumber Brantas, East Java, Indonesia. Approximately five grams of compost were serially diluted and vortexed in 30 ml distilled water. Thereafter, plated 100 µL of solution and streaked onto nutrient agar (NA) medium at 28 °C for 16 h. NA medium was coated by 100 folded serial dilutions of cyclohexylamine. After 16 hours, the bacterial isolates were purified by subculture onto the new NA medium three times until single colonies were obtained. The selected bacteria isolated were cultured in M63 medium to see the growth rate of each bacteria. Further OD measurements (560 nm) were carried out at intervals of 0 to 72 hours.

Cytokinin production

To evaluate cytokinin production, the selected isolates were cultured in M63 medium. After 48 h of incubation, the cultures were analyzed for the presence of cytokinin using gas chromatography/mass spectrometry (GC-MS).

Research stages for metabolite screening using GC-MS

The observation of metabolite screening was measured by modified method by Goubet et al (2021). 100 uL of sample was prepared by centrifuging at 9500 rpm for 5 minutes to obtain the supernatant, which was diluted with methanol (MeOH) to a final volume of 1 mL in a microtube and vortexed until homogenized. The homogenized supernatant was transferred into a GC vial and injected into the GC-MS system using a HP-5MS UI column (30 m length, 0.25 mm I.D., 0.25 µm film thickness, 325/350 °C max temperature). Helium served as the carrier gas with an injector temperature of 230 °C, a split ratio of 50, and a front inlet flow of 1.00 mL/min. The MS transfer line and ion source were set to 250 °C and 200 °C, respectively, with a mass detection range of 40–500 amu. The column program began at 60 °C for 2 minutes, ramped to 280 °C at 10 °C/min, and was held for 8 minutes. The volume sample was 50 uL. Metabolite screening targeted cytokinin compounds, known for their critical role in cell division and plant growth regulation, by matching retention times and mass spectra with reference libraries (e.g., NIST). This approach allowed for the identification and quantification of cytokinins, providing valuable insights into their biosynthesis and potential application in plant growth enhancement.

Data analysis

Detected compounds are identified by matching their retention times and mass spectra with reference libraries (e.g., NIST). The results provide a list of metabolites, including relevant plant growth hormones such as cytokinins, which are recorded for further analysis. Growth rates and cytokinin production were analyzed to identify the most promising rhizobacterial isolates for improving plant tolerance. Data were visualized using graphs and diagrams for better interpretation.

RESULTS AND DISCUSSION

The concentration of various phytohormones

The phytohormones measured include kinetin riboside, zeatin riboside, kinetin 7 N glucoside, trans-zeatin glucoside, kinetin 9 N glucoside, kinetin, cis-zeatin, trans-zeatin, and 6-benzylaminopurine.

The highest concentration of kinetin riboside was observed in the IRT1 10^{-3} PS isolate, followed by IRT3 10^{-4} CO, with IRT3 10^{-2} PS having the lowest concentration. The results were 2.03%, 1.48% and 1.55%, respectively (Figure 1).

A similar trend was observed where zeatin riboside was found in IRT1 10^{-3} PS having the highest concentration by 1.39%, but the difference between isolates IRT3 10^{-2} PS and IRT3 10^{-4} CO was smaller compared to other cytokinin derivatives. The highest concentrations for Kinetin 7 N glucoside and trans-zeatin glucoside were found in IRT1 10^{-3} PS, while IRT3 10^{-2} PS consistently showed lower concentrations across all phytohormones. The concentrations were 2.79%, 2.08% and 2.14%, respectively. Thus, the IRT1 10^{-3} PS isolate again demonstrated the highest concentration of kinetin, followed by comparable levels in IRT3 10^{-4} CO and IRT3 10^{-2} PS. Both cis-zeatin and trans-zeatin follow a similar trend, where IRT1 10^{-3} PS consistently showed the highest levels, while IRT3 10^{-2} PS and IRT3 10^{-4} CO displayed moderate variations. The lowest concentration of 6-Benzylaminopurine was observed for this phytohormone across all isolates, with IRT1 10^{-3} PS still maintaining the highest level compared to the other isolates (Figure 1).

The study showed that the IRT1 10^{-3} PS strain showed higher phytohormone concentrations compared to other bacterial strains. This indicates that this strain has a higher impact on growth response and stress tolerance through the secretion of phytohormones produced. Fahad et al. (2015) explained that phytohormones in plants play an important role in increasing plant growth and stress tolerance, especially under drought stress conditions. Drought stress significantly affects plant survival and overall agricultural production.

Based on Figure 1 demonstrated that cytokinin derivatives produced by three bacterial strains include kinetin riboside, zeatin riboside, kinetin

7 N glucoside, trans zeatin glucoside, kinetin 9 N glucoside, kinetin, cis Zeatin, trans Zeatin and 6-Benzylaminopurine. These compounds are cytokinin compounds and their derivatives. Liu et al. (2020) stated that cytokinins play an important role in cell differentiation and their influence in mitigating stress conditions. Other compounds of cytokinins such as kinetin riboside play a role in regulating oxidative stress by helping to reduce cell damage due to free radicals caused by environmental stress. Not only that, kinetin riboside also plays a role in modulating plant metabolic pathways by increasing protein synthesis to reduce stress in plants (Naseem et al., 2020). Mok (2019) explained that kinetin riboside is also involved in regulating stomatal closure so that it can maintain water needs in drought conditions. The next cytokinin compound is zeatin riboside, this compound plays a role in increasing plant growth through stimulation of root and shoot cell division and widening of leaf area that supports the plant photosynthesis process (Schiller and Magnitskiy, 2019). In addition, Zaheer et al. (2024) explained the role of zeatin riboside in increasing tolerance to abiotic stress is by increasing antioxidant activity which can reduce plant damage due to free radicals that occur during stress.

In this study also showed the concentration of cytokinin compounds, namely kinetin and kinetin 7 N glucoside. Kinetin is another form of cytokinin, while kinetin 7 N glucoside is a conjugated form of kinetin. According to Hamayun et al. (2015) and Pokorna et al. (2020), both compounds are involved in stimulating the formation of roots and shoots that encourage increased plant growth. In stressful conditions, both compounds play a role in maintaining hormone stability and reducing high free radicals in plants.

Thus, two other cytokinin compounds are trans zeatin and zeatin glucoside. Both of these compounds have the same function in cell

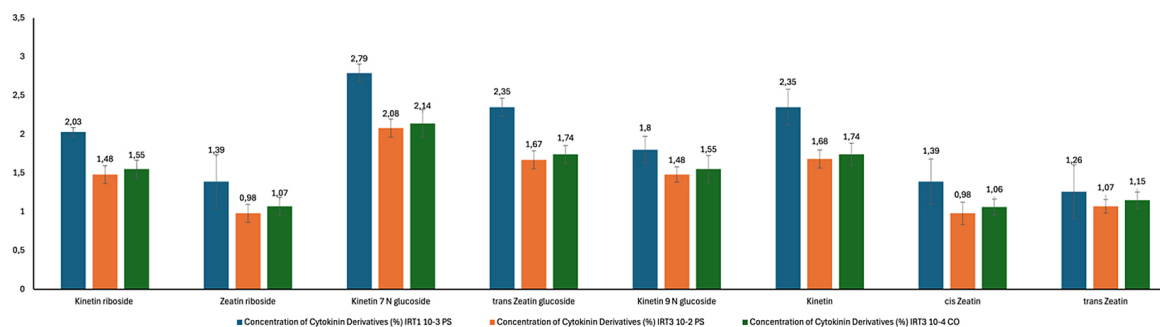


Figure 1. The concentration of cytokinin derivatives

division and their role in plant growth (Pokorna et al., 2020; Hallmark et al., 2020). Hoyerová and Hošek (2020) explained that trans zeatin is more biologically active than zeatin glucoside. The next compound is cis zeatin and benzylaminopurine (BAP), both of these compounds are involved in the important process of plant growth in the cell division process. Between these two compounds, BAP shows a more effective compound compared to cis zeatin (Jameson, 2019). Lu et al. (2015) added that this happens because BAP is more stable in various conditions and is easily translocated and absorbed by plants.

The structure of cytokinin compounds and their derivatives can be seen in Figure 2. In kinetin compounds, the chemical structure is identical to the purine structure in its side chain bound to nitrogen 6 (N6). Kinetin riboside has a ribose bond in its chemical structure, while kinetin 7 N glucoside and kinetin 9 N glucoside bind to glucose on sides number 7 and 9. The chemical structure of zeatin also binds to purine but is modified at position 6 and has a hydroxyl group at position 7. Zeatin is modified into cis zeatin and trans zeatin, both of which have the same position modification at position 6 but with different configurations, cis zeatin with cis isomers while trans is with trans isomers. In addition, 6-benzylaminopurine (BAP) has a zylamin group at position 6

so that it shows a different structure compared to zeatin and kinetin. Understanding the structure of phytohormones or their derivative compounds can contribute to overcoming environmental conditions that are not suitable for plant growth, such as reducing plant stress levels or supporting plant growth (EL Sabagh et al., 2022).

The metabolic pathways of several cytokinin compounds have different pathways (Figure 3). Zeatin is formed from adenine which is involved in cell division and growth. In other forms, zeatin is isomerized into cis zeatin which can occur naturally in plants. On the other hand, zeatin also undergoes modification by adding ribose to zeatin riboside. Zeatin riboside has lower biological activity than zeatin itself. Not only the addition of ribose, zeatin also undergoes glycolysis or the addition of glucose to trans zeatin glucoside which functions as zeatin storage. Zeatin and its modifications are important for promoting cell division and shoot formation (Tank et al., 2015).

Kinetin is also one of the cytokinins that can also undergo several modifications. Kinetin plays an active role in triggering cell division, stimulating shoot growth and contributing to inhibiting aging in plant cells. Kinetin undergoes glycolysis with ribose to become kinetin riboside which functions as storage and transportation in plant cells. Kinetin can also be conjugated with

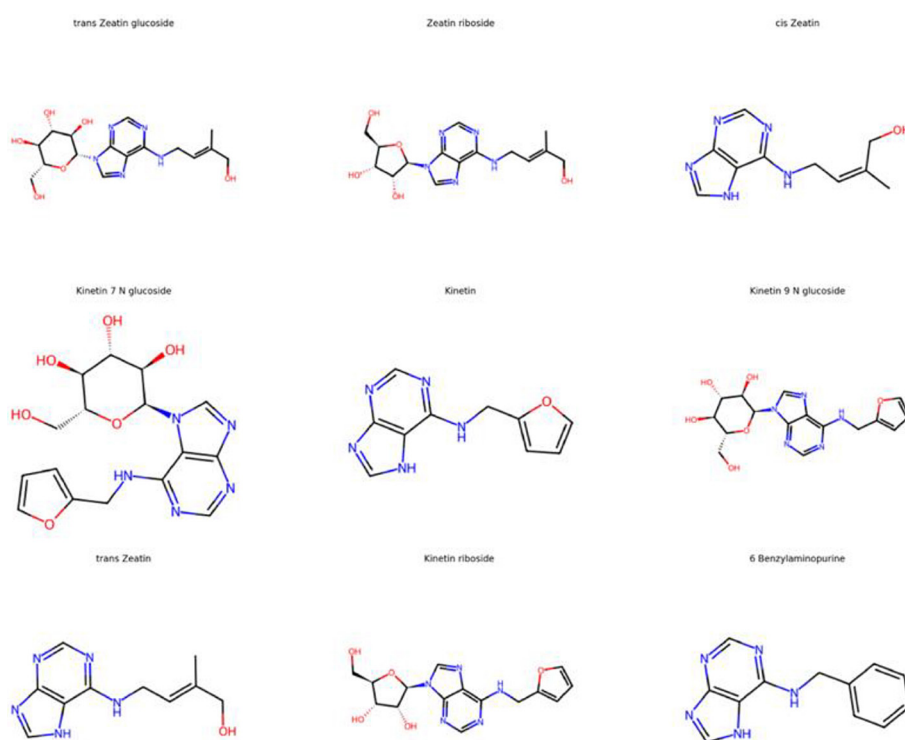


Figure 2. Chemical structure of cytokinins and their derivatives

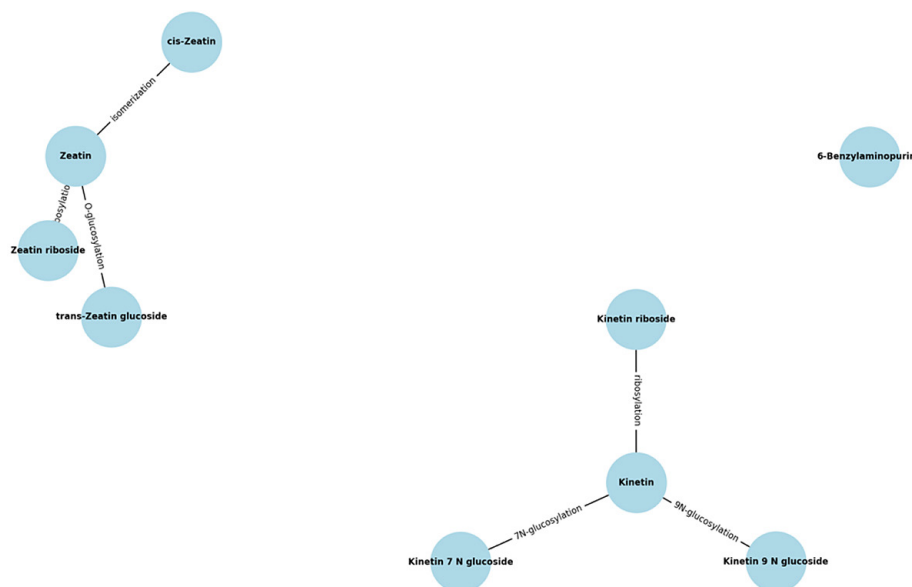


Figure 3. The metabolic pathways and interconversion processes between different cytokinin molecules

glucose to become kinetin glucoside. Kinetin 7 N glucoside and kinetin 9 N glucoside have glucose addition at nitrogen (N) position in parts 7 and 9. Overall, kinetin and its modifications play an crucial role in cell division and growth regulation (Li et al., 2018).

BAP is a part of cytokinin that is often used in tissue culture research to stimulate cell division and shoot proliferation. Although BAP is not included in the endogenous cytokinin metabolism of plants, BAP is widely used in laboratory experiments and research to observe cell division, shoot growth and inhibit plant aging (Matušková et al., 2020). Figure

4 showed that cytokinins are involved in mediating abiotic stress. In stressful conditions, such as drought, salinity or high temperatures, plants produced a lot of cytokinins that function to regulate plant growth and development. Cytokinins are absorbed by plants through the xylem and distributed throughout the plant. Not only that, cytokinins are also well absorbed by roots or leaves. Furthermore, at the signal perception stage, receptors in plant cells such as AHK proteins bind cytokinins which are then involved in signal transduction. At this stage, protein kinases become active and plant cells can adapt to the stress conditions that occur.

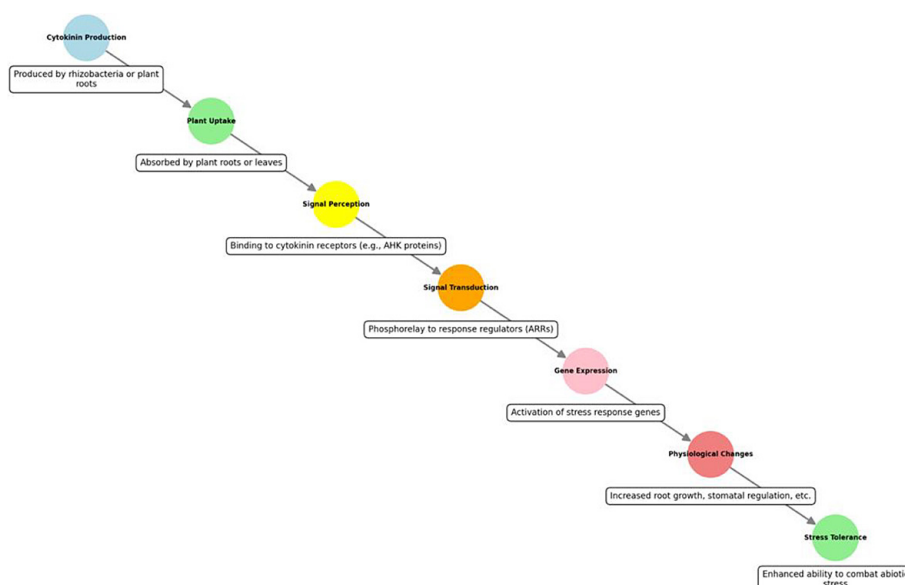


Figure 4. Cytokinin-mediated abiotic stress response

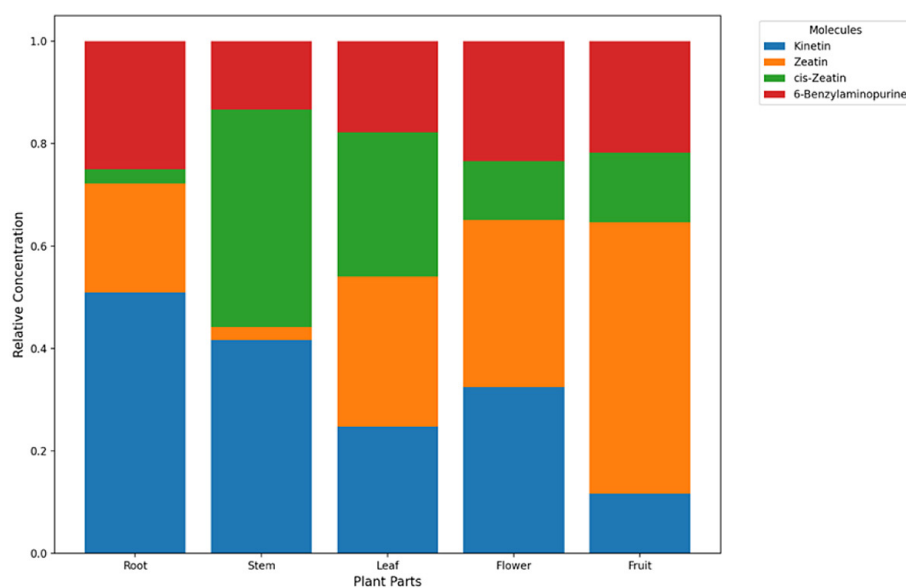


Figure 5. Overview of metabolites profiling

The plant's response to stress affects physiological changes in plants such as root elongation, stomata opening and closing and morphological changes in leaves and other plant organs. Ultimately, overall, these cytokinins can mediate plants in adapting to abiotic stress conditions (Gujjar and Supaibulwatana, 2019).

In this study, metabolites rhizobacteria profiling such as kinetin, zeatin, cis-zeatin and 6-benzylaminopurine showed different proportions in each part of the plant (Figure 5). In the roots, the highest kinetin molecules were found compared to other molecules such as zeatin, BAP, and cis zeatin. This indicated that kinetin plays an important role in regulating cell division and cell differentiation in the roots. Kinetin plays an active role in supporting the transportation and distribution of plant nutrients. Zeatin and 6-benzylaminopurine showed moderate proportions, indicating that in the roots these 2 molecules also play an important role in cell division and plant growth regulation. On the other hand, cis zeatin demonstrated a very small proportion compared to other molecules, meaning that cis zeatin is less active in plant root function (Figure 5). Bielach et al. (2017) stated that plants can regulate cytokinin needs according to physiological needs and specific conditions of plant parts.

In the stems, the concentration of zeatin and kinetin showed the largest proportion of molecules compared to 6-benzylaminopurine and cis zeatin. This indicated that both molecules play a very important role in stimulating stem elongation

and overall stem growth. The proportion of kinetin is still high, but not as much as the proportion of kinetin in the roots. This confirmed that the compounds and hormonal profiles in the roots can move from the roots to the stems according to the dominant needs in the stems (Figure 5). Li et al. (2022) added that the difference in the proportion of molecules in the stems suggest that plants can specifically regulate the physiological needs for stem development and growth.

In the leaves, the cytokinin profile demonstrated the same cytokinin compounds, where zeatin, cis zeatin and kinetin had an even proportion when compared to the roots and stems of the plant. This revealed that the three compounds play an active role in regulating leaf growth, plant photosynthesis, and overall plant health (Figure 5). According to Mandal et al. (2022), the three molecules are also involved in modulating stress responses, especially in drought stress conditions. Although the proportion of 6-benzylaminopurine did not show as many results as the other three molecules, its role is very important in stimulating growth and inhibiting aging in plants, especially in leaves.

In flowers, the proportion of zeatin and kinetin also identified the most abundant cytokinin compounds compared to the other two molecules, cis zeatin and 6-benzylaminopurine (Figure 5). This confirmed that zeatin and kinetin were dominantly involved in promoting cell division during flowering and fruit formation. According to Zakharova et al. (2022), zeatin plays an important

role in stimulating flower growth and development. Zeatin is also involved in regulating the maturation of the fertilization process in plants.

In fruit growth, it was found that the concentration of zeatin showed the highest results compared to kinetin, cis zeatin and 6-benzylaminopurine. The zeatin molecule dominates the distribution of cytokinins compared to the other 3 molecules. This suggested that zeatin plays an active role in stimulating growth, supporting cell and tissue division, and stimulating fruit ripening. Teribia et al. (2016) emphasized that zeatin contributes to supporting the metabolic process during fruit ripening.

To understand the possible mechanism and experimental simulation of cytokinin under both normal and drought stress condition, there are hypothetical changes in cytokinins distribution in plant parts such as roots, stems, leaves, flowers and fruits of plants. These changes include changes in the proportion of cytokinin modifications such as the amount of zeatin under normal conditions, while decreasing under drought conditions in the fruit of the plant (Figure 6). This suggests that the distribution of cytokinin molecules plays an important role in regulating plant growth and mediating plants in increasing tolerance to environmental stress conditions.

Specifically, under normal conditions, the concentration of cytokinins is distributed evenly throughout the plant, including the roots, stems, leaves, flowers, and fruits (Figure 6). In plant roots, the concentration of kinetin is more dominant than the other 3 cytokinins, namely zeatin, cis-zeatin, and 6-benzylaminopurine. These assume

that kinetin plays a very important role in regulating growth and cell differentiation in plant roots. Under drought conditions, the amount of kinetin in the roots is still high, while zeatin and cis zeatin are getting lower. Surprisingly, the concentration of 6-benzylaminopurine also shows a higher concentration than under normal conditions. This confirms that kinetin and 6-benzylaminopurine are cytokinin molecules that play an important role in drought adaptation in plant roots.

In plant stems, under normal conditions the distribution of cytokinin molecules from high to low is respectively kinetin, cis zeatin, 6-benzylaminopurine, and zeatin (Figure 6). Kinetin and cis zeatin show the highest proportion under normal conditions. Under drought conditions, cis zeatin experiences a decrease in concentration while 6-benzylaminopurine shows a higher portion compared to under normal conditions. This indicates that 6-benzylaminopurine under normal conditions does not play a specific role compared to under stress conditions, especially under drought conditions. Ghaleh et al. (2020) emphasized that 6-benzylaminopurine plays an important role in regulating and mediating environmental stress and increasing its tolerance.

In the leaves, under normal conditions, the distribution of cytokine molecules is more even in terms of concentrations of kinetin, zeatin, cis zeatin and 6-benzylaminopurine. While under drought conditions, the concentration of 6-benzylaminopurine shows a higher portion than under normal conditions, while the concentration of zeatin is lower. These suggested that 6-benzylaminopurine is very important in mediating

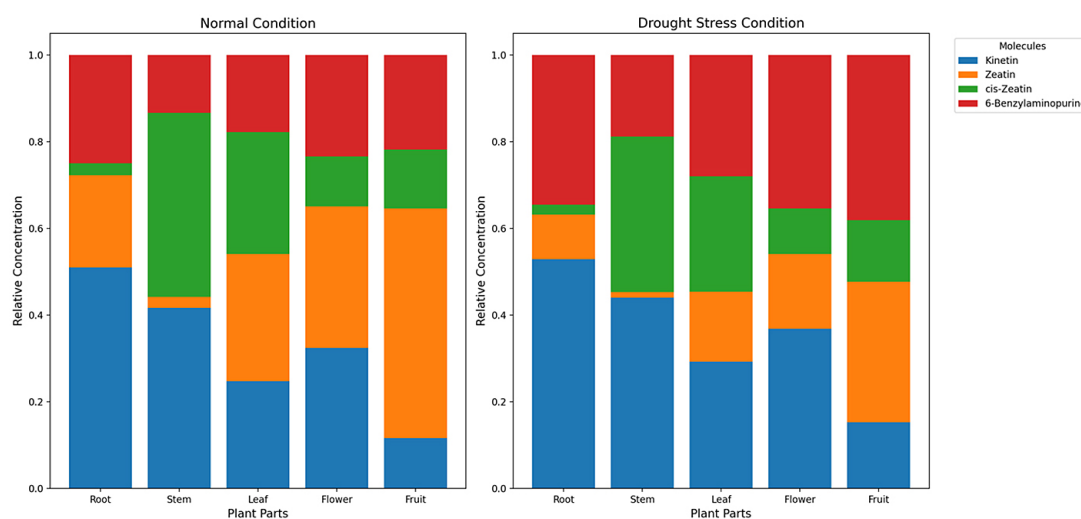


Figure 6. Hypothetical changes in cytokinin distribution under drought stress

drought stress in plant leaves. Sihotang et al., 2024 explained that 6-benzylaminopurine is involved in modulating drought stress in many plants.

Furthermore, in the flower section, the portion of kinetin and zeatin showed the highest value under normal conditions, while during drought conditions 6-benzylaminopurine showed a high value. Not only that, surprisingly the concentration of zeatin showed a decrease during drought conditions. This indicates that zeatin plays an active role in cell differentiation in plant flowers under normal conditions while high 6-benzylaminopurine plays an important role in mediating stress tolerance. In the fruit section, the concentration of zeatin dominates compared to other cytokinin molecules. This shows that zeatin plays a very important role in regulating the growth and development of plant fruits as a whole both under normal conditions and drought stress. Uniquely, the concentration of 6-benzylaminopurine also shows a high portion compared to normal conditions. These revealed that 6-benzylaminopurine kind of cytokinin involved in growth response and plant tolerance during stress conditions. Overall, cytokinins and their modifications play a very important role in modulating abiotic stress (Pavlů et al., 2018).

Many studies have shown that cytokinins play an active role in cell division and tolerance to abiotic stress. Figure 7 shows the biosynthesis of cytokinins in rhizobacteria. Specifically, this mechanism involves several inactive enzymes becoming active again. The key enzymes involved in activating cytokinins are labeled in

green (Figure 7), namely LOG (LONELY GUY), CYP735A, and IPT (Isopentenyl Transferase). In maintaining hormonal balance in plants, the LOG enzyme is catalyzed to break down adenine derivatives to produce active cytokinins (Bielach et al., 2017). CYP735A is an enzyme that plays an active role in inducing inactive cytokinins to become active again, and is involved in the biosynthesis of trans-zeatin (tZ) (Sýkorová, 2007). DMPP (Dimethylallyl Pyrophosphate) and iP (adenine isopentenyl) are important precursors in the formation of cytokinin structures related to plant cell division (Soderberg, 2000). Not only that, iPR and its derivatives are also involved in forming more complex cytokinin structures. Thus, two other enzymes, namely cis zeatin (cZ) and trans zeatin (tZ) play a role in the response to stress and cell differentiation, where cZ focuses on increasing tolerance to stress, while tZ plays an important role in plant growth and development. ATP is needed by plants in all biochemical reactions of plant cells (Jameson, 2023).

PGPR is a rhizobacteria that plays a major role in increasing plant growth and tolerance to various unfavorable environmental conditions such as biotic and abiotic stress (Maulidah et al., 2021). Figure 8 showed how PGPR interacts with plant growth hormones and compounds involved in increasing tolerance to environmental stress. PGPR produces antioxidants to ward off free radicals and increase tolerance to biotic and abiotic stress. PGPR produces IAA to promote plant root growth. ACC Deaminase is also produced by PGPR to reduce plant ethylene levels

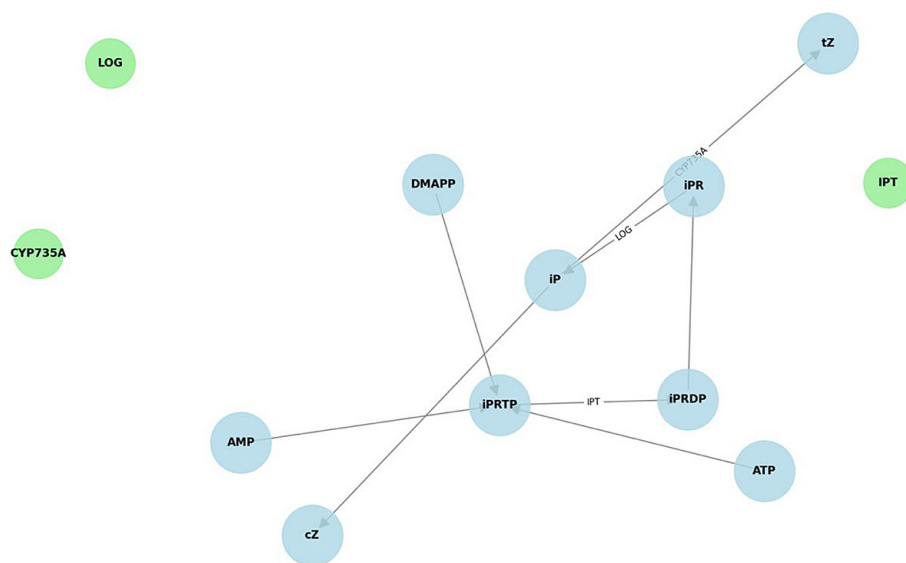


Figure 7. The cytokinin biosynthesis pathway in rhizobacteria



Figure 8. The rhizobacteria-mediated plant protection against abiotic stresses

and help reduce environmental stress. To maintain ion homeostasis, PGPR produces osmolytes in sufficient quantities so that plants can survive better during osmotic stress. PGPR can also produce siderophores which play an important role in maintaining iron deficiency in the soil and increasing resistance to stress. Overall, PGPR plays an important role in promoting plant growth and plant tolerance to biotic and abiotic stress (Kurniawan and Chuang, 2022; Ikhwan et al., 2022).

CONCLUSIONS

This study successfully demonstrated that rhizobacteria isolated from tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) plants have the potential as a source of cytokinins that can increase plant tolerance to abiotic stress. Ten rhizobacteria isolates tested showed good growth patterns in M63 media, and most of them were able to produce various forms and derivatives of cytokinins, such as Trans Zeatin, cis Zeatin, and Kinetin. Isolate IRT1 10^{-3} PS stood out with the highest concentration of various phytohormones, showing a significant positive impact on plant growth and stress response. These findings underline the importance of utilizing rhizobacteria for increasing plant resistance to unfavorable environmental conditions. Through

cytokinin production, rhizobacteria can increase plant physiological activities, including photosynthetic efficiency and root growth, which in turn supports sustainable agricultural productivity. Further research is needed to understand the specific mechanisms of interaction between rhizobacteria and plants, in order to develop more effective bioinoculants to support agriculture under abiotic stress.

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