INHIBITORY EFFECTS OF PROPICONAZOLE ON RICE ROOT DEVELOPMENT AND ABILITIES OF BRASSINOSTEROID AND AUXIN IN ALLEVIATING ITS EFFECTS

Mr. Watcharapong Wimonchaijit

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ผลการยับยั้งของโพรพิโคนาโซลต่อการเจริญของรากข้าวและความสามารถของบราสสิโนสเตอรอยด์และออกซินในการลดผลกระทบ

นายวัชรพงศ์ วิมลไชยจิต

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Thesis Title	INHIBITORY EFFECTS OF PROPICONAZOLE ON RICE ROOT DEVELOPMENT AND ABILITIES OF BRASSINOSTEROID AND AUXIN IN ALLEVIATING ITS EFFECTS
By	Mr. Watcharapong Wimonchaijit
Field of Study	Botany
Thesis Advisor	Assistant Professor Juthamas Chaiwanon, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

Dean of the FACULTY OF SCIENCE

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

Chairman

(Associate Professor SEHANAT PRASONGSUK, Ph.D.)

Thesis Advisor

(Assistant Professor Juthamas Chaiwanon, Ph.D.)

Examiner

(Professor MANIT KIDYOO, Ph.D.)

Examiner

(Assistant Professor ANCHALEE CHAIDEE, Ph.D.)

External Examiner

(Jareerat Chunthawodtiporn, Ph.D.)

วัชรพงศ์ วิมลไชยจิต : ผลการยับยั้งของโพรพิโคนาโซลต่อการเจริญของรากข้าวและความสามารถของบราสสิโนสเตอรอยด์และออกซินในการลดผลกระทบ. (INHIBITORY EFFECTS OF PROPICONAZOLE ON RICE ROOT DEVELOPMENT AND ABILITIES OF BRASSINOSTEROID AND AUXIN IN ALLEVIATING ITS EFFECTS) อ.ที่ปรึกษาหลัก : ผศ. ดร.จุฑามาศ ชัยวนนท์

โพรพิโคนาโซล เป็นสารเคมีที่ใช้กำจัดเชื้อรา นิยมใช้ในการเกษตรเพื่อลดความเสียหายจากการก่อโรคของเชื้อรา การใช้โพรพิโคนาโซลอย่างต่อเนื่องอาจทำให้เกิดปัญหาด้านสิ่งแวดล้อมได้ เนื่องจากโพรพิโคนาโซลมีเวลาครึ่งชีวิตที่ยาว สามารถคงค้างอยู่ในดินหลังจากการใช้งานได้เป็นเวลานาน ดังนั้นจึงมีความจำเป็นในการศึกษาผลกระทบของการใช้โพรพิโคนาโซลเพื่อเป็นข้อมูลเพิ่มเติมในการประเมินถึงความเสี่ยงในการใช้สารชนิดนี้ การศึกษานี้แสดงให้เห็นว่า โพรพิโคนาโซลยับยั้งการเจริญเติบโตของข้าวในระยะต้นกล้า ซึ่งต้นกล้าของข้าวที่ได้รับโพรพิโคนาโซลแสดงอาการของการเจริญเติบโตที่ลดลงอย่างเห็นได้ชัด ได้แก่ ความสูงและน้ำหนักที่ลดลง ลักษณะทางสัณฐานวิทยาของใบที่ผิดปกติ และการเจริญของรากที่ถูกยับยั้ง การศึกษาที่ผ่านมาพบว่า โพรพิโคนาโซลมีฤทธิ์ในการยับยั้งชีวสังเคราะห์ของบราสสิโนสเตอรอยด์ในพืช ดังนั้นผลการศึกษานี้จึงสนับสนุนความสำคัญของบราสสิโนสเตอรอยด์ในควบคุมการเจริญที่เหมาะสมของพืช นอกจากนี้โพรพิโคนาโซลยังมีผลยับยั้งการเจริญของรากแขนงอีกด้วย รากข้าวที่ได้รับโพรพิโคนาโซลมีความหนาแน่นของรากแขนงบนรากปฐมภูมิที่ลดลงเมื่อเทียบกับรากข้าวที่ไม่ได้รับสารชนิดนี้ โดยโพรพิโคนาโซลมีผลยับยั้งการเจริญออกมาของปุ่มกำเนิดรากแขนงจากรากปฐมภูมิและการยืดตัวของรากแขนง ผลการศึกษาเพิ่มเติมพบว่า การให้กรด 1-แนฟทาลีนแอซีติก ซึ่งเป็นออกซินสังเคราะห์ สามารถลดผลกระทบของโพรพิโคนาโซลต่อการยับยั้งการเจริญของรากแขนงได้ เนื่องจากรากข้าวที่ได้รับโพรพิโคนาโซลคู่กับกรด 1-แนฟทาลีนแอซีติก มีความหนาแน่นของรากแขนงของรากปฐมภูมิไม่แตกต่างจากความหนาแน่นของรากแขนงของรากปฐมภูมิของรากข้าวปกติที่ไม่ได้รับสารใดเลย แสดงว่าโพรพิโคนาโซลทำให้เกิดความผิดปกติในกลไกของออกซินที่ควบคุมการเจริญของราก

สาขาวิชา พฤกษศาสตร์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWOR D: propiconazole, lateral root, brassinosteroid, auxin

Watcharapong Wimonchaijit : INHIBITORY EFFECTS OF PROPICONAZOLE ON RICE ROOT DEVELOPMENT AND ABILITIES OF BRASSINOSTEROID AND AUXIN IN ALLEVIATING ITS EFFECTS. Advisor: Asst. Prof. Juthamas Chaiwanon, Ph.D.

Propiconazole is commonly used as a fungicide to minimize crop loss caused by various fungal diseases in many crop plants, including rice. Propiconazole has the potentials to deteriorate the environmental problems due to its chemical properties that it is highly persistent in soil after application, suggesting that the constant use of propiconazole could be problematic. Hence, the evaluation of its effects on plant growth and development is necessary for the assessment of future use of propiconazole. Here, we demonstrated that propiconazole had inhibitory effects on plant growth. Treatments of propiconazole resulted in shoot dwarfism, abnormal leaf morphology, reduced plant biomass, and inhibited root growth, further supporting the importance of brassinosteroid for regulating optimal plant growth and development due to the ability of propiconazole to inhibit brassinosteroid biosynthesis. In addition, propiconazole reduced emerged lateral root density by inhibiting the emergence of lateral root primordia and lateral root elongation. Treatments of 1-naphthaleneacetic acid (NAA), a synthetic auxin, could rescue lateral root density in the propiconazole-treated plants to the untreated level, suggesting that auxin-mediated regulation of lateral root development was defective in the propiconazole-treated plants.

Field of Study:	Botany	Student's Signature .....
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Watcharapong Wimonchaijit

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CHAPTER I
INTRODUCTION

Thai rice (*Oryza sativa* L.) is one of the most important agronomic crops of Thailand because Thailand is one of the world leading rice exporters and consumes rice as the main food. Therefore, rice is an important factor relating to food and economic security of the country (Arunrat and Pumijumnong 2015). Importantly, Thai local rice has high genetic variation, which is useful resource for research on rice (Kladmook, Kumchoo et al. 2012).

Root system is the underground part of plants which plays pivotal role in water uptake and nutrient acquisition from soil. In addition, plant roots not only provide storage function and mechanical support for the aboveground parts, but also serve as the major interface that perceives and responds to external stimuli, triggered by both biotic and abiotic factors (Lynch 1995, Smith and De Smet 2012). The characteristic of the root system is one of the essential factors determining root system performance and thus affects plant yield (Lynch 1995, Smith and De Smet 2012). Previous studies demonstrated that modifying root traits has been shown to be a promising way to improve crop performance resulting in increasing yield (Zhan, Schneider et al. 2015). In rice, lateral roots emerge from their main root axes, which are primary root and crown roots. The branching and growth of lateral roots account for a large proportion of the total root length and surface area, and thus provide a great potential for water uptake and soil exploration of the root system (Gowda, Henry et al. 2011). Therefore, the characteristics of lateral roots is an important factor affecting the root system performance.

Propiconazole is a triazole compound which has been widely used as a systemic fungicide in many crop plants including rice to reduce the loss of plant productivity caused by fungal diseases such as rice false smut disease (Fan, Yang et al. 2016). Propiconazole is commonly used via foliar application to control diseases in rice at the growing stage (Pan, Cheng et al. 2018). The constant use of many pesticides including propiconazole caused the environmental pollution. The accumulation of propiconazole was detected in the topsoil of paddy rice field (Braun, Sebesvari et al. 2018). Moreover, propiconazole is highly persistent in soil because its degradation after application was minimal (Kim, Beaudette et al. 2002, Kim, Shim et al. 2003). Therefore, the use of propiconazole would be one of the factors contributing to a significant environmental contamination. In addition, propiconazole has been reported to have inhibitory effects on plant growth. Propiconazole-treated plants had significantly shorter plant height (Best, Hartwig et al. 2014). Moreover, a previous study demonstrated that propiconazole is a potent specific inhibitor of the biosynthesis of brassinosteroid (Hartwig, Corvalan et al. 2012). This suggests that the effect of propiconazole on plant growth is necessary to be evaluated.

Plant growth and developmental processes are tightly regulated by their phytohormones such as brassinosteroid and auxin. Brassinosteroid is a plant-specific steroid hormone that promotes root growth at low concentrations, but inhibits root growth at high concentrations (Clouse, Langford et al. 1996, Tong, Xiao et al. 2014). The loss-of-function mutant of brassinosteroid receptor had short root phenotype (Hacham, Holland et al. 2011). Brassinosteroid also positively regulated the development of lateral roots at low concentration via the promotion of auxin transport in *Arabidopsis thaliana* (Bao, Shen et al. 2004, Gupta, Singh et al. 2015). Auxin is another plant hormone which plays an indispensable role in the initiation and subsequent development of lateral roots. The number of lateral roots was decreased in *Osiaa11* and *Osiaa13* mutants, which have the defect in the auxin signalling, due to the inhibition of lateral root primordia initiation (Kitomi, Inahashi et al. 2012, Zhu, Liu et al. 2012). Moreover, the knock-down mutation of *OsAUX3*, an auxin influx carrier, also had the lower number of lateral roots and lateral root primordia (Wang, Qiao et al. 2019). These studies demonstrated that brassinosteroid and auxin are required for normal root growth and development.

In this study, the effects of propiconazole on rice root growth and development were observed with the focus on lateral root development and interaction between brassinosteroid and auxin treatment.

Objectives

1) To investigate the effects of propiconazole rice root growth and development of lateral roots

2) To evaluate specific effects of propiconazole on rice root response to brassinosteroid and auxin treatment

CHAPTER II
LITERATURE REVIEW

2.1 Food security and the importance of rice

Rice is not only one of the main economic crops of Thailand due to being the major rice exporter (Arunrat and Pumijumnong 2015), but also the main staple food for half of the world's population. Thus, factors that affect rice production will impact the world's food security (Arunrat and Pumijumnong 2015, Singha, Dong et al. 2019, Firdaus, Leong Tan et al. 2020). To cope with the high demands of rice, one way that farmers can apply is to use pesticides to enhance crop yield and increase their income (Wang, Chu et al. 2018). However, the use of pesticides can worsen the environmental problem (Riise, Lundekvam et al. 2004, Braun, Sebesvari et al. 2018, Wang, Chu et al. 2018, Tao, Jia et al. 2021).

2.2 Propiconazole

Propiconazole (1-(2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl)-1H-1,2,4-triazole, $C_{15}H_{17}Cl_2N_3O_2$, figure 1) is a triazole compound and classified as a systemic fungicide, which can be transported from the site of its application to other plant organs (Ju, Li et al. 2020). Propiconazole is extensively used in agricultural fields to control a wide range of fungal diseases in various cereal plants, such as rice false smut disease, rice blast, dirty panicle diseases, leaf spot, and powdery mildew (Sun, Thai et al. 2005, Fan, Yang et al. 2016, Kongcharoen, Kaewsalong et al. 2020). Propiconazole is highly persistent in soil due to its long half-life, which is about 214 days in soil (Braun, Sebesvari et al. 2018). Previous studies reported that propiconazole residues were detected in soil even after more than 300 days of application, depending on soil types (Kim, Shim et al. 2003, Riise, Lundekvam et al. 2004, Satapute and Kaliwal 2018). In addition, a report from the observation in Vietnam provided evidence that the accumulation of propiconazole was detected in paddy rice fields with the average concentration at 9.6 μg kg^{-1} and the maximum concentration at 18.8 μg kg^{-1} (Braun, Sebesvari et al. 2018) and soil in wheat fields in China with the average concentration at 2.6 μg kg^{-1} and the maximum concentration at 40.5 μg kg^{-1} (Tao, Jia et al. 2021). Therefore, the continuous use of propiconazole can be problematic due to its potential contamination problems if it accumulates until it reaches the threshold concentrations detrimental to plants (Bradley D, Carol A et al. 2003).

Propiconazole has the chemical ability to interfere with steroid biosynthesis in fungi by inhibiting lanosterol 14α-demethylase (CYP51), which is a crucial enzyme for the biosynthesis of ergosterol, a component of the fungal plasma membrane (Yoshida and Aoyama 1991). In plants, the study of how plants uptake propiconazole in wheat roots indicated that propiconazole is mainly absorbed via apoplastic pathways (Ju, Li et al. 2020). Similar to fungi, propiconazole also has adverse effects on plant growth. The propiconazole-treated plant showed strong retarded growth with shorter plant height and root length in maize and *Arabidopsis* (Hartwig, Corvalan et

al. 2012). Further investigations demonstrated that propiconazole inhibits plant sterol biosynthesis by specifically interfering with the CYP450 enzyme(s), catalyzing the rate-limiting step(s) of brassinosteroid biosynthesis (Best, Hartwig et al. 2014, Oh, Matsumoto et al. 2016, Bajguz, Chmur et al. 2020). Therefore, propiconazole has been used as a potent and alternative specific inhibitor of brassinosteroid biosynthesis with lower cost and easier accessibility than other brassinosteroid biosynthesis inhibitors such as brassinazole and BRZ2001 (Rozhon, Akter et al. 2019).

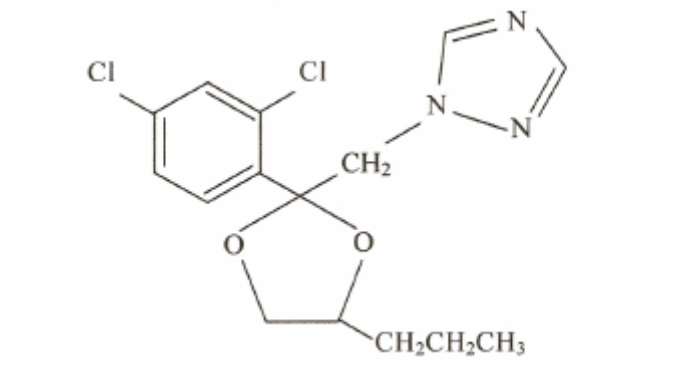

Figure 1 Chemical structure of propiconazole (Andreea, Ochiuz et al. 2010)

2.3 Plant root system

Root system is the underground part of plants that plays various pivotal roles in plant physiological functions, not only water uptake, nutrient acquisition, storage, mechanical support, but also being the major interface perceiving and responding to external stimuli, both abiotic and biotic factors surrounding the rooting medium (Lynch 1995, Smith and De Smet 2012). Numerous researches underlie the importance of root-related traits that significantly impact plant productivity and crop yield. For example, rice cultivars with the deep root system showed more tolerance to severe drought stresses and had higher crop yields than those with the shallow root system (Uga, Sugimoto et al. 2013). Another example also demonstrated that reducing lateral root density improved drought tolerance by reducing the metabolic costs for soil exploration and thus enhancing rooting depth that allowed deeper soil exploration leading to increased water acquisition from soil under drought (Zhan, Schneider et al. 2015), suggesting that modifying root traits is another way to improve crop performance.

2.3.1 Types of the root system

The root system of flowering plants is classified into two main types (figure 2) (Osmont, Sibout et al. 2007, Sparks and Benfey 2017).

2.3.1.1 Primary root system (tap root system) is the root system that consists of two main types of roots, which are primary root and lateral root. Primary root is the root that originates from the embryonic radicle and further develops into the central root axis of the primary root system, which can grow

indeterminately in order to explore deeper soil layers. Lateral roots subsequently develop and emerge from the primary root axis, and lateral roots themselves can also branch further orders of lateral roots similar to the primary root. This type of root system is commonly found in dicotyledons.

2.3.2.2 Fibrous root system is the root system that the primary root has determinate growth and the essential functions for a period of time during seedling development. Later on, the primary root undergoes senescence and eventually degenerates. This type of root system has post-embryonic roots that are derived from non-root tissue, such as stem base and stem node, called adventitious roots (Atkinson, Rasmussen et al. 2014). Adventitious roots can also form lateral roots from their root axes and are responsible for the root system functions replacing the primary root at the later developmental stages. This type of root system is commonly found in monocotyledons.

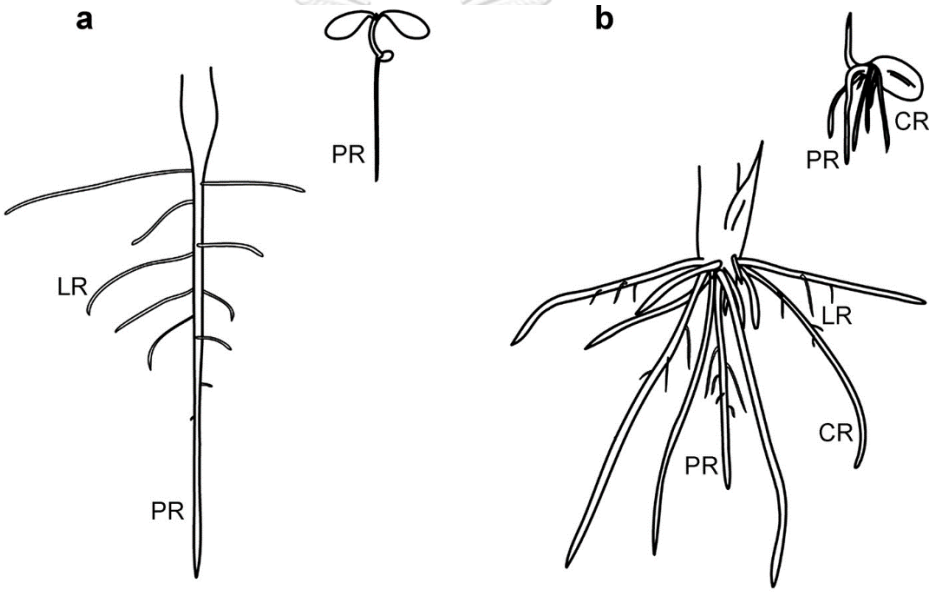

Figure 2 Types of the plant root system. A). Primary root system (tap root system) B). Fibrous root system (Osmont, Sibout et al. 2007). PR, primary root; LR, lateral root; CR, crown root.

2.4 Rice root system

Rice root system is the fibrous root system and comprises four types of roots (figure 3) (Gowda, Henry et al. 2011).

2.4.1 Seminal root is the embryonic roots originating from the radicle. In rice, there is only one seminal root, which is the primary root.

2.4.2 Mesocotyl roots are the post-embryonic roots that emerge from mesocotyl region.

2.4.3 Crown roots are the post-embryonic roots that emerge from the base of the rice stem. This type of root is the main component of the rice fibrous root system.

2.4.4 Lateral roots are the post-embryonic roots derived from existing roots, which are the seminal root, mesocotyl roots, crown roots, and lateral roots themselves. There are two types of lateral roots in rice, which are small lateral roots and large lateral roots. Only large lateral roots can branch higher orders of lateral roots.

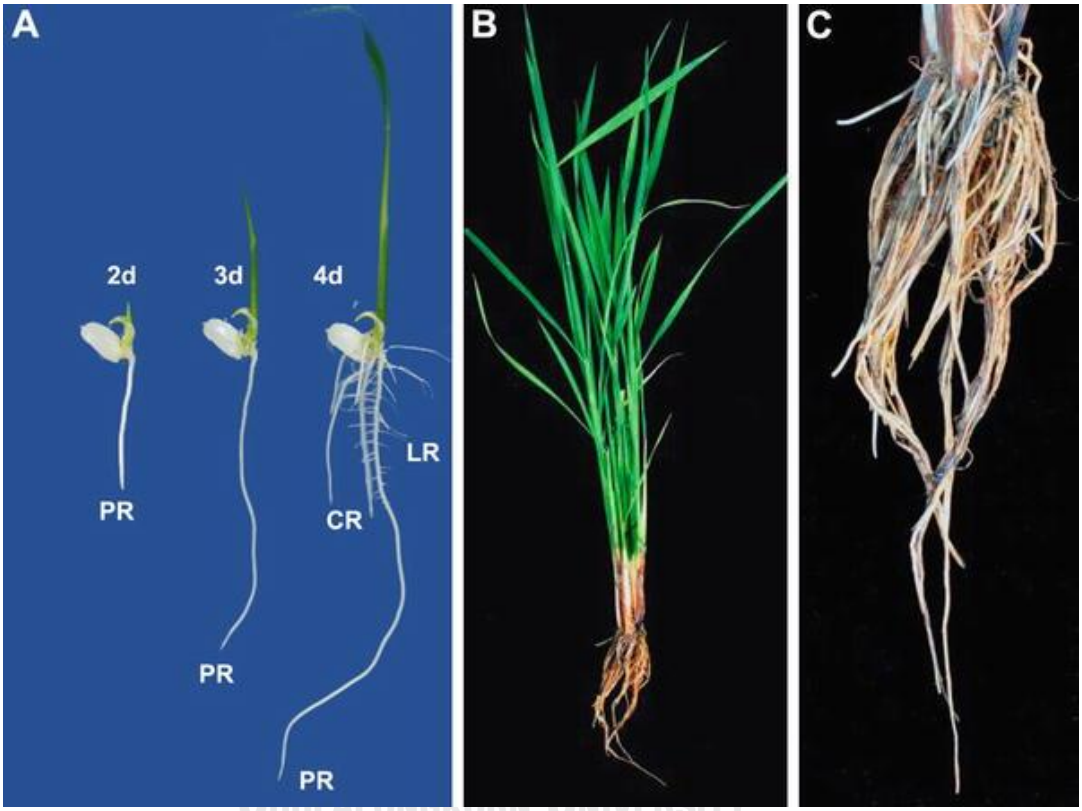

Figure 3 Rice root system. A) Roots of rice seedling from two-four days after germination. B-C) Roots of rice a 60-day-old rice plant the fibrous root system. (Xu and Hong, 2013) PR, primary root; CR, crown root; LR, lateral root.

2.5 Root developmental zones

Root developmental zones can be divided into three zones in the longitudinal axis (figure 4) (Verbelen, De Cnodder et al. 2006, Petricka, Winter et al. 2012, Barrada, Montané et al. 2015).

2.5.1 Meristematic zone comprises mitotically-active cells that divide and produce new cells for further root growth and development, which the most actively-divided cells reside in the proximal part of the meristematic zone, called the root apical meristem (RAM). Mitotic competence of the newly-produced cells from the RAM decreases when they gradually grow and exit the meristematic zone with increasing competence for the rapid cell elongation in the elongation zone. These cells reside in the distal part of the meristematic zone and partly overlap with the elongation zone, called the basal meristem or transition zone.

2.5.2 Elongation zone consists of the cells that stop dividing and rapidly elongate.

2.5.3 Differentiation zone or maturation zone is the zone that cells reach their final cell size and maturely differentiate, which is noticeably characterized when root hairs elongate from the epidermal cells.

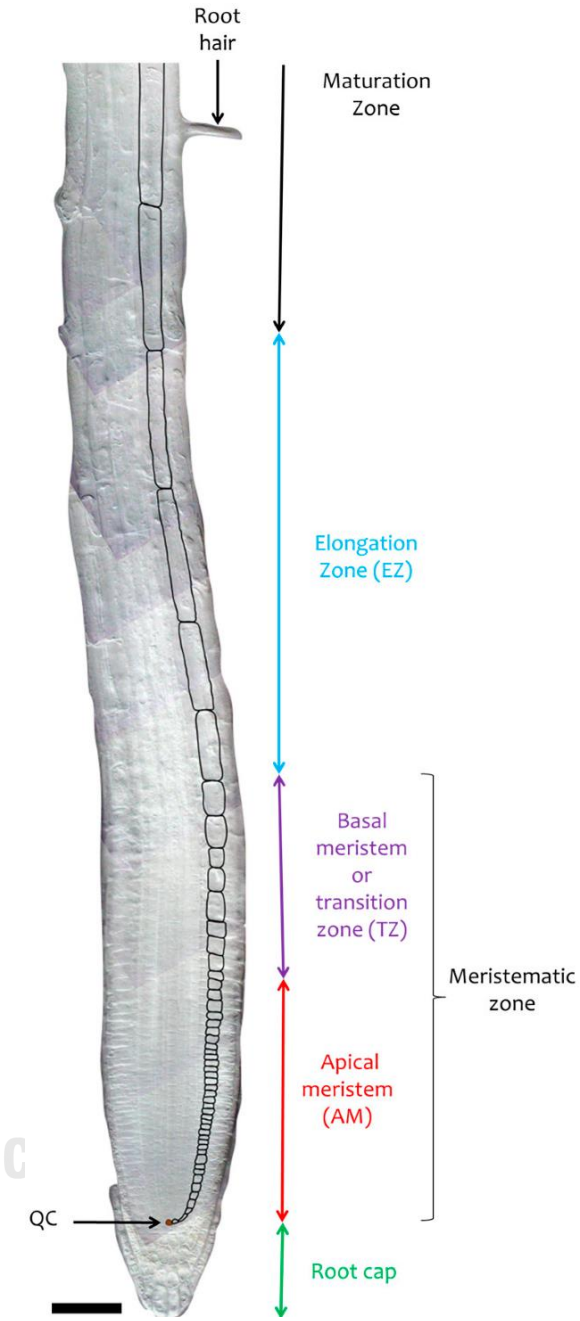

Figure 4 Root developmental zones. Modified from Barrada, Montané et al. (2015)

2.6 Development of lateral roots

The development and branching of lateral roots constitute a considerable proportion of the whole root system structure, especially total root length and surface area, thus essentially determining the capability of the root system for soil exploration and the uptake of soil water and nutrient (Gowda, Henry et al. 2011). The developmental processes initiate at the proximal part of the root tip as observed in *Arabidopsis* that lateral root formation begins in the transition zone and the elongation zone and shows the acropetal growth pattern that younger lateral roots are found in

the near root tip part and older lateral roots are found in the more shootward part of roots (figure 5) (Dubrovsky, Gambetta et al. 2006). The development of lateral roots is well-described in the *Arabidopsis* model plant that has the simple root structure, comprising of each one layer of epidermis, cortex, and endodermis, and the innermost stele including vascular tissues and one layer of pericycle (Malamy and Benfey 1997), which can be divided into four steps according to Du and Scheres (2017).

2.6.1 Priming or lateral root positioning is the preparatory step consisting of mechanisms that position, specify, and activate groups of pericycle cells to acquire the competence to be the lateral root founder cells (LRFCs), which further divide and develop into the lateral root primordia (LRP). These processes occur mostly in the transition zone and the elongation zone of the root tip (Du and Scheres 2017). In *Arabidopsis*, LRFCs are derived from the pericycle cells adjacent to the xylem pole, known as xylem-pole-pericycle cells (Malamy and Benfey 1997, Dubrovsky, Rost et al. 2001).

2.6.2 Lateral root initiation is the process that the swelling and the nuclear polarization of the specified LRFCs occur in the differentiation zone of the root tip (Vermeer, Wangenheim et al. 2014, Du and Scheres 2017). Then, LRFCs undergo the first asymmetrical and anticlinal division, which is perpendicular to the main root axis, to form the LRP (figure 7) (Du and Scheres 2017, Laskowski and ten Tusscher 2017),

2.6.3 Lateral root morphogenesis is the process that involves the formation of LRP from the founder cells following the initiation process with subsequent cell divisions and patterning of the root apical meristem and tissues of newly-formed lateral roots (Du and Scheres 2017). This process can be divided into seven stages (figure 6) (Malamy and Benfey 1997, Dubrovsky, Rost et al. 2001, Petricka, Winter et al. 2012). At stage I, the single layer of LRP is formed as the result of the first division of the specified LRFCs as described in 2.6.2. At stage II, the LRP further divides periclinally, resulting in the two layers of the LRP, which are outer layer (OL) and inner layer (IL). At stage III, another periclinal division is observed in OL, forming the three layers of the LRP, and the IL cells also divide periclinally, generating the LRP with four layers (two OLs and two ILs) at stage IV. These developmental processes occur inside the overlying endodermis. Then, during stage V-VII, the LRP expands radially and penetrates through the overlying tissues, which are endodermis, cortex, and epidermis, with the series of both anticlinal and periclinal divisions, resulting in forming the dome-shaped or mushroom-shaped LRP. The root tip structure of the LRP begins to develop maturely at stage VI. The stele of LRP is noticeably observed, and the LRP is about to emerge out of the epidermis at stage VII and finally grows out of the parent root at stage VIII, defined as lateral root emergence (Malamy and Benfey 1997, Casimiro, Beeckman et al. 2003).

2.6.4 Lateral root emergence is the process that occurs simultaneously with the morphogenesis of the LRP. The developing LRP must penetrate through the overlying tissues and eventually breaks out of the parental root. Mechanisms that facilitate the emergence coincide with the development of the LRP. Studies in *Arabidopsis* revealed that the overlying endodermal cells underwent morphological changes and eventually shrank (Vermeer, Wangenheim et al. 2014). In addition, the Casparian strip, which is the ring-like hydrophobic barrier surrounding the endodermal cells and restricts the direct apoplastic movement of external water and solutes into the stele, was locally degraded to accommodate the outgrowth of the LRP (Vermeer, Wangenheim et al. 2014, Banda, Bellande et al. 2019). The cell wall of the overlying cortical and epidermal cells was also loosened with the induction of cell wall remodeling enzymes, resulting in the loss of cell adherence to enhance the LRP emergence (figure 7) (Swarup, Benková et al. 2008, Péret, Middleton et al. 2013, Zhu, Shao et al. 2019).

The developmental processes of lateral roots in cereal plants, including maize (*Zea mays*) (figure 8), and rice (figure 9), are similar to *Arabidopsis* in terms of the developmental sequences as described above (Orman-Ligeza, Parizot et al. 2013, Ni, Shen et al. 2014, Yu, Gutjahr et al. 2016). However, there are distinguishable differences. Cereal plants have a more complex root anatomy. In mature roots of rice, there are up to ten layers of ground tissues in cortex in rice depending on root types (figure 10) (Coudert, Périn et al. 2010). Hence, the developing LRP in cereal roots must pass through thicker layers of the overlying tissues than *Arabidopsis*. Moreover, lateral roots in cereal plants are originated from phloem-pole-pericycle cells, not the xylem-pole-pericycle cells in the same way as *Arabidopsis*. In addition, endodermal cells also participate in forming the LRP, which is the origin of lateral root cap cells in the mature root tip, while other tissues originate from pericycle (Kawata and Shibayama 1965, Rebouillat, Dievart et al. 2009).

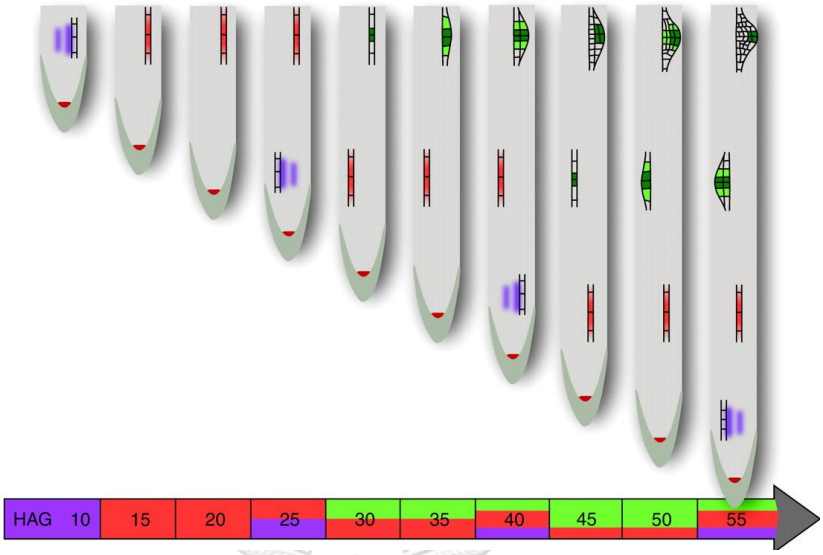

Figure 5 The development of the formation of lateral root primordia from the priming to the emergence is illustrated in spatial and temporal manners in *Arabidopsis* (Overvoorde, Fukaki et al. 2010). HAG, hours after germination.

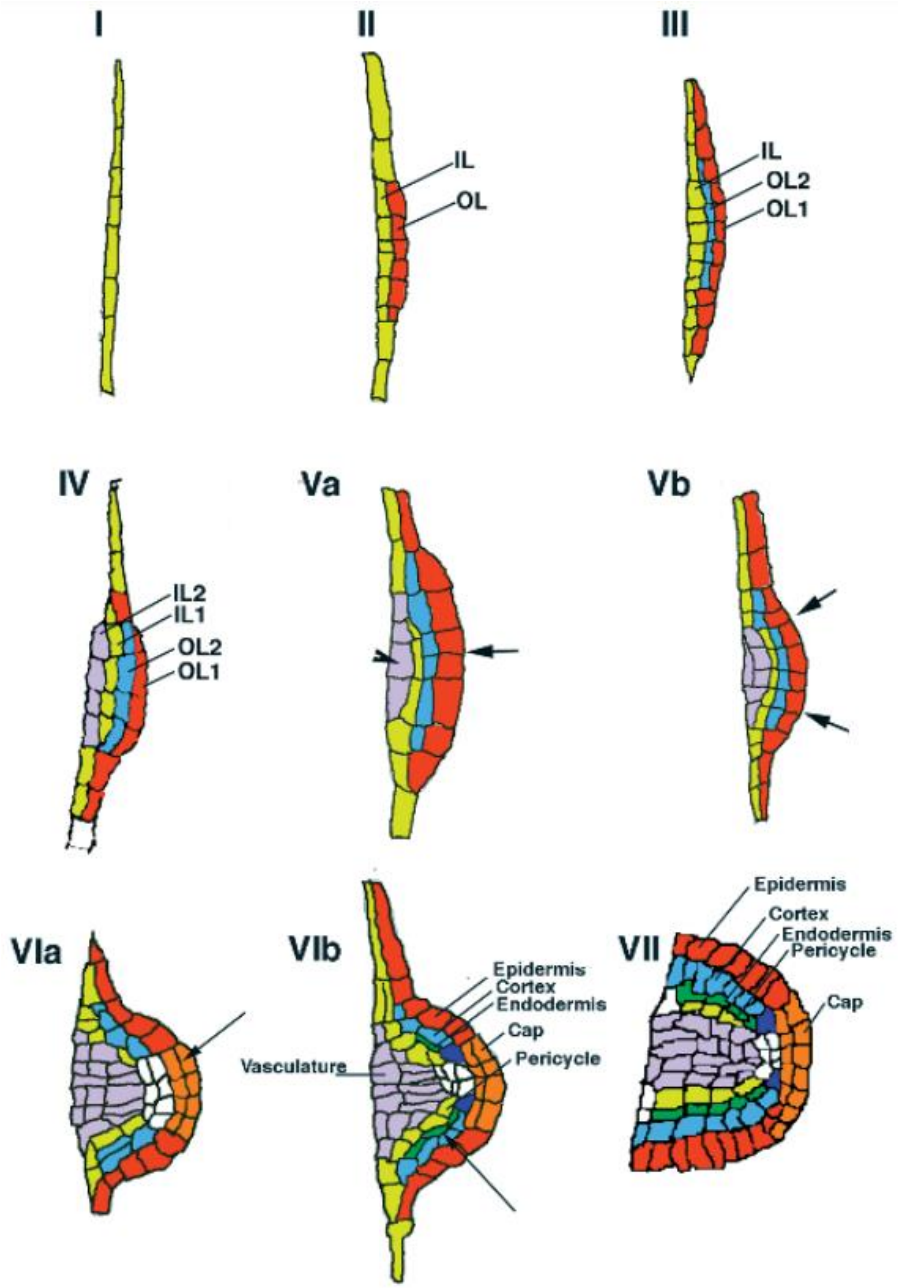

Figure 6 Developmental stages of the lateral root morphogenesis from stage I to VII in *Arabidopsis* (Malamy and Benfey 1997). OL, outer layer; IL, inner layer.

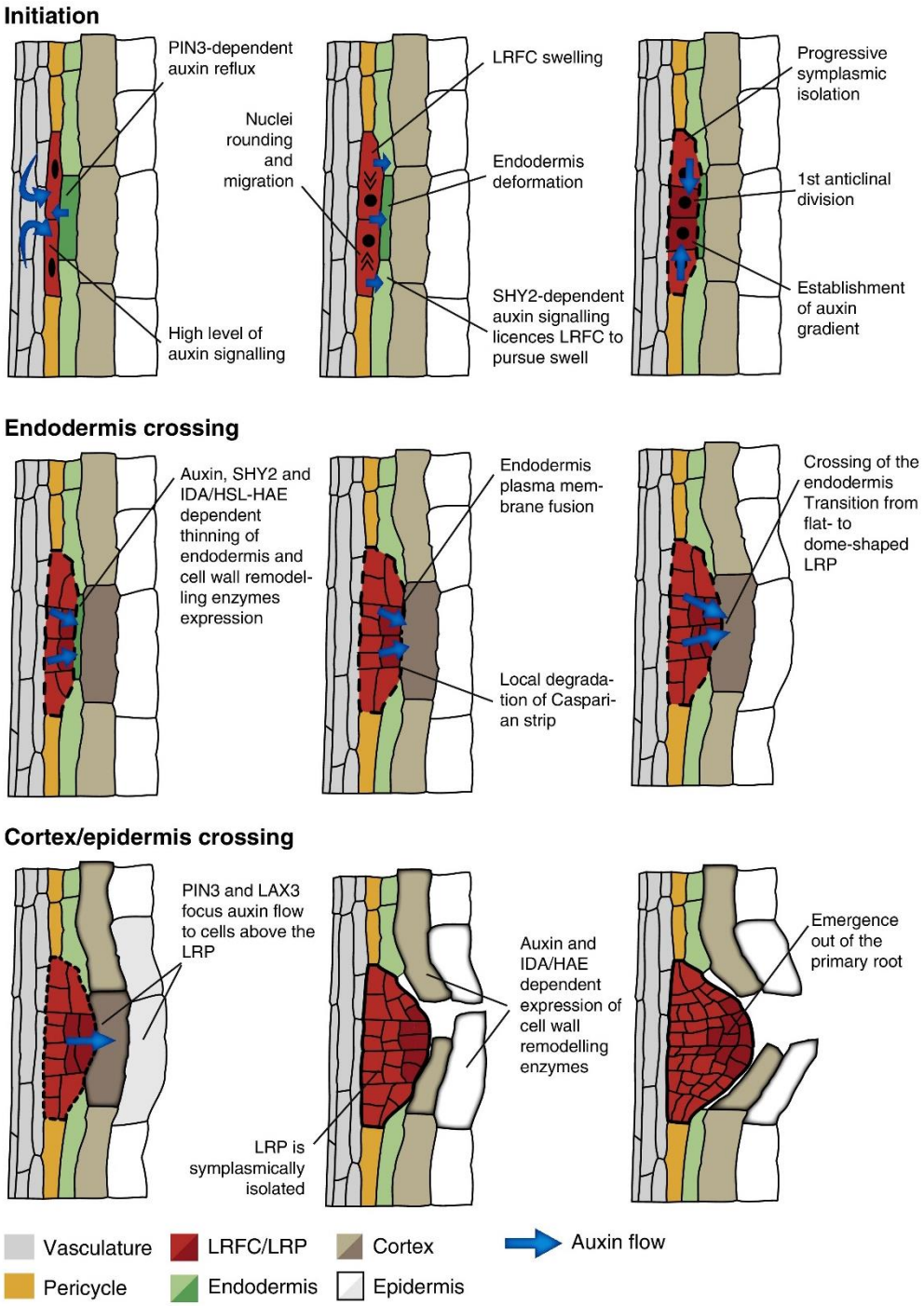

Figure 7 Summary of the auxin-mediated mechanisms during the development of lateral roots from the initiation to the emergence of lateral root primordia in *Arabidopsis* (Vilches-Barro and Maizel 2015). LRFC, lateral root founder cells; LRP, lateral root primordia.

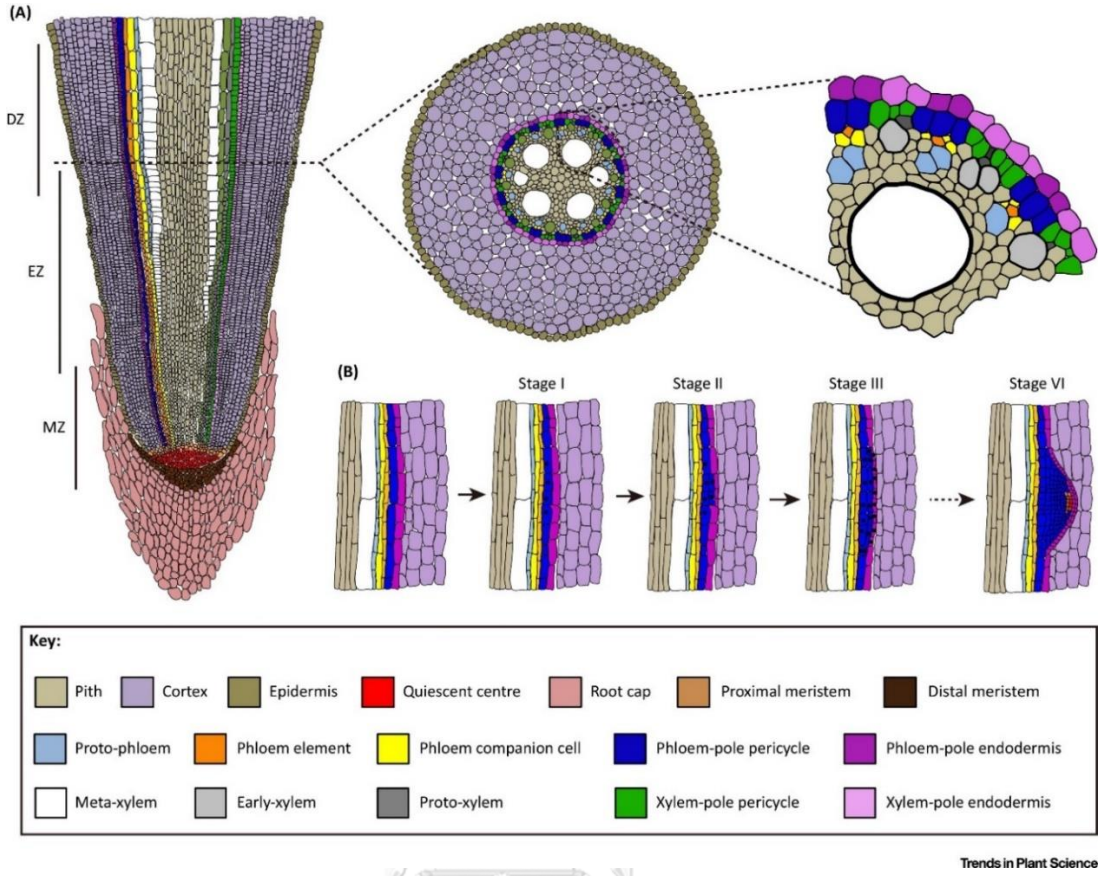

Figure 8 Cellular organization and lateral root development in maize roots. A). Cellular organization of maize root tip in the longitudinal axis and cross-section from the differentiation zone. B). The development of lateral root primordia starts from the initiation to stage VI. At stage III, endodermal cells divide and participate in the organogenesis of the developing primordia (Yu, Gutjahr et al. 2016). MZ, meristematic zone; EL, elongation zone; DZ, differentiation zone.

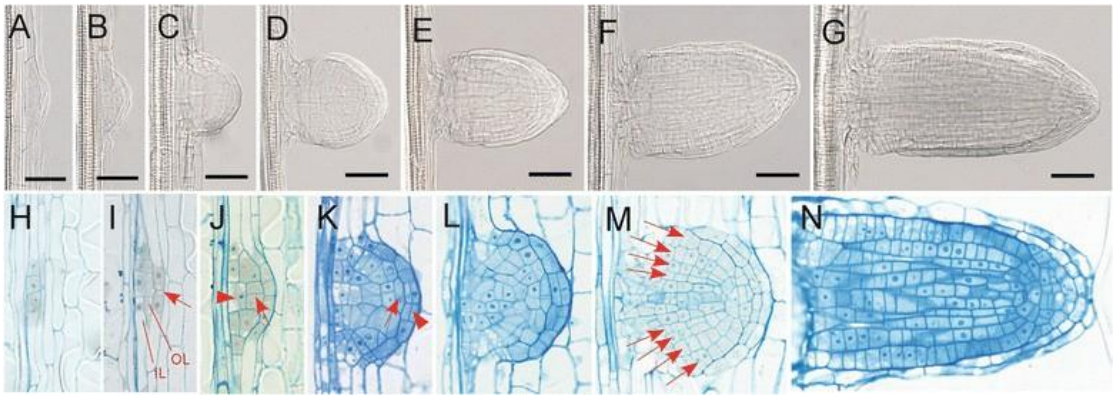

Figure 9 Lateral root development in rice roots. A-G). The development of lateral root primordia after the removal of the overlying cortex under a compound microscope. H-J). Sections of different stages of the developing lateral root primordia. I). The red arrow indicated the periclinal division of endodermal cells in contrast to Arabidopsis. K-M). The developing lateral root primordia progressed to the dome-shaped or mushroom-shaped primordia. M). The red arrow indicated the four layers of the cells surrounding stele. N). The section of emerged mature lateral root (Ni, Shen et al. 2014). OL, outer layer; IL, inner layer. Scale bars in A-G). = 50 μm

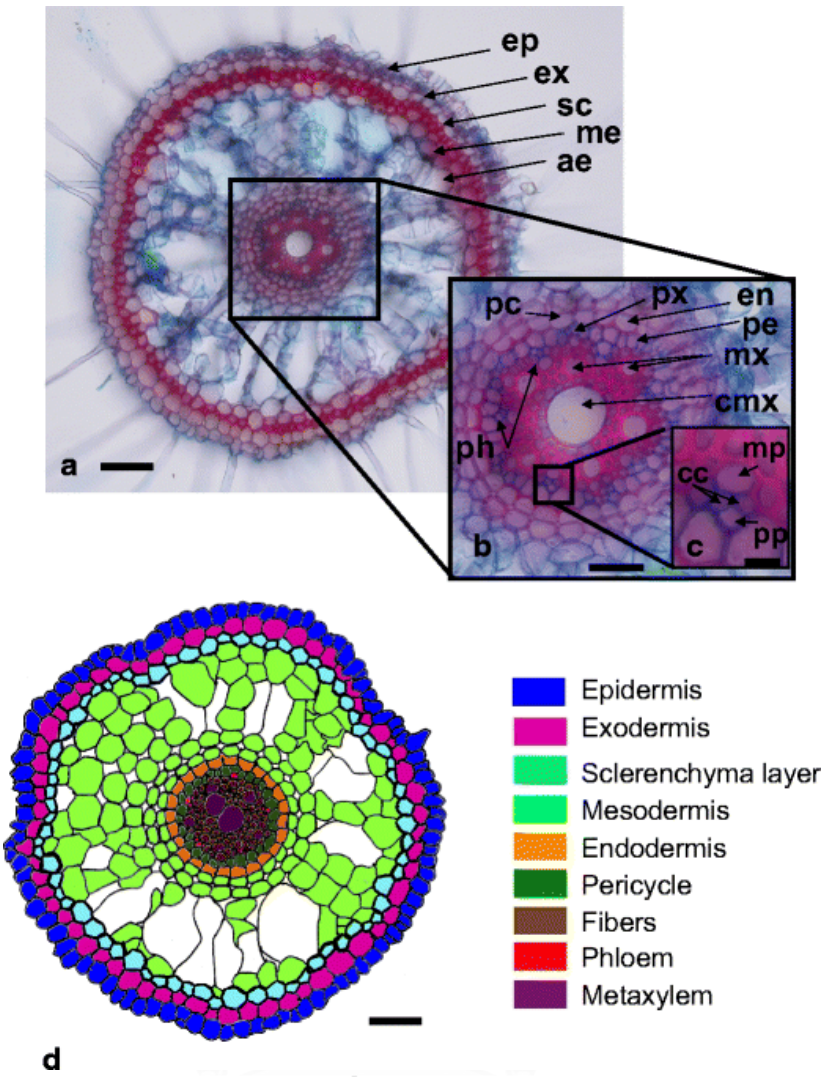

Figure 10 Radial anatomy of the primary root of rice. A-C). Transverse section from about 2 cm from the root tip. D). A representative graphics of cellular organization of the transverse section of the primary root of rice (Rebouillat, Dievart et al. 2009). ep, epidermis; ex, exodermis; sc, sclerenchyma layer; me, mesodermis; ae, aerenchyma lacunae; en, endodermis; pe, pericycle; mx, metaxylem; cmx, central metaxylem; pp, protophloem; cc, companion cells; mp, metaphloem; pc, endodermis passage cell; px, protoxylem; ph, phloem. Scale bars in A). = 50 μm, B). = 25 μm, C). = 5 μm, and D). = 50 μm.

2.7 Auxin

Auxin is one of the plant hormones that play indispensable roles in plant growth and development of which auxin has been known to be the master regulator. Previous researches have shown that auxin involves in embryogenesis, tissue specification, cell expansion, meristem integrity, gravitropic responses, adventitious rooting from non-root tissues, and lateral root developments (Petricka, Winter et al. 2012, Olatunji, Geelen et al. 2017, Han, Adamowski et al. 2021). The biosynthesis, signalling, and transport mechanisms orchestrate the proper action of auxin (Overvoorde, Fukaki et al. 2010, Olatunji, Geelen et al. 2017, Brumos, Robles et al. 2018). In lateral root development, auxin participates in the regulation of all developmental stages, starting from the priming to the emergence (Figure 7 and 11) (Lavenus, Goh et al. 2013, Santos Teixeira and ten Tusscher 2019). Previous studies demonstrated that changes in the biosynthesis, signalling, and transport of auxin affected the development of lateral roots.

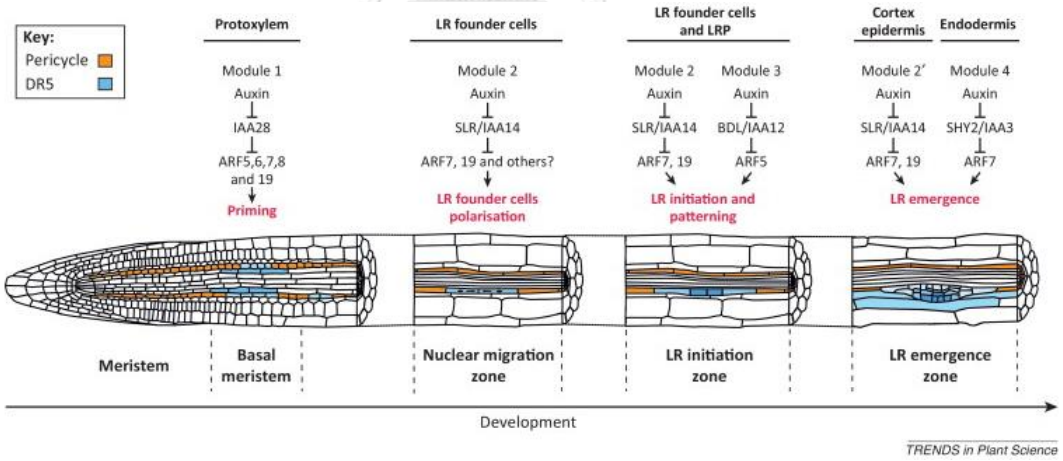

Figure 11 The auxin-mediated control of lateral root development via the auxin signalling pathway in *Arabidopsis* (Lavenus, Goh et al. 2013).

2.7.1 Auxin biosynthesis

Indole-3-acetic acid (IAA) is the most naturally predominant auxin in plants (Sreevidya, Hernandez-Oane et al. 2010, Korasick, Enders et al. 2013, Swarup and Bhosale 2019). It is mainly biosynthesized from tryptophan via the TAA-YUC pathway consisting of two main steps, catalyzed by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and

YUCCA (YUC), respectively (Mashiguchi, Tanaka et al. 2011, Korasick, Enders et al. 2013, Olatunji, Geelen et al. 2017) (Figure 12). Loss of function of the auxin biosynthesis genes dramatically inhibits lateral root development. Auxin contents in shoot and root were reduced by half in *ostaa1* (*fib1, fish bone1*) rice mutants, and the phenotypes of the mutant showed almost no emerged lateral roots and lower number of crown roots, compared to the wildtype (Yoshikawa, Ito et al. 2014, Zhang, Li et al. 2018). Similar phenotypes were also observed in the transgenic rice expressing the antisense of *OsYUCCA1*, which inhibited the expression of *OsYUCCA1*, displayed severe shoot dwarfism and defective root development (Yamamoto, Kamiya et al. 2007). While, the overexpression of *OsYUCCA1* led to the overproduction of roots both crown roots and lateral roots (Yamamoto, Kamiya et al. 2007, Zhang, Li et al. 2018).

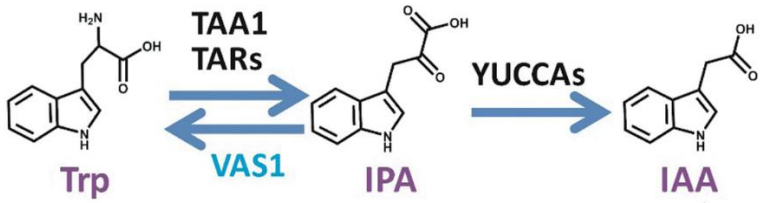

Figure 12 The biosynthetic pathway of auxin. Modified from Wang, Zhang et al. (2017).

2.7.2 Auxin signalling

Plant cells perceive auxin by its receptor TIR1/AFB (TRANSPORT INHIBITOR RESPONSE 1/AUXIN-RELATED FBOX PROTEINS), localized within cells (Badescu and Napier 2006). The binding of auxin to TIR1 leads to the degradation of Aux/IAA (AUXIN/INDOLE-3-ACETIC ACID) proteins that repress the action of Auxin Response Factor (ARF) transcription factors, resulting in the activation of the expression of auxin-responsive genes, which are the downstream targets of ARFs (Figure 13) (Das, Weijers et al. 2021). Defects in the signalling components cause drastic effects on the lateral root development. The initiation of lateral root primordia was inhibited in the gain-of-function *OsIAA11* mutants of which the auxin signalling was impaired (Zhu, Liu et al. 2012). The phenotypes of the *Osiaa11* mutants showed the insensitivity to exogenous auxin treatments and had no lateral root primordia and emerged lateral roots in the homozygous mutants (Zhu, Liu et al. 2012). Similar effects on the inhibition of lateral root development were also observed in other gain-of-function *OsIAA* mutants, for instance, *OsIAA13* (Kitomi, Inahashi et al. 2012) and *OsIAA23* (Jun, Gaohang et al. 2011). Loss of function in *OsARF* genes also negatively affected the

development of lateral roots, which *Osarf19* mutant had approximately 30% lower number of emerged lateral roots (Zhang, Wang et al. 2015).

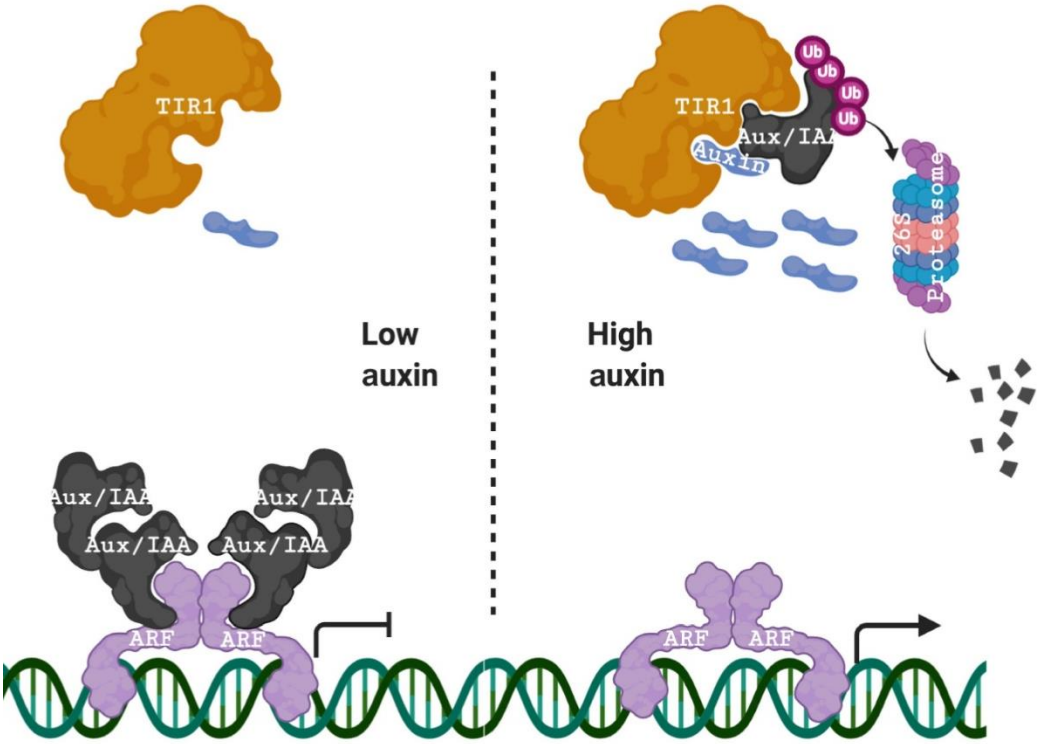

Figure 13 The signalling pathway of auxin (Das, Weijers et al. 2021).

2.7.3 Auxin transport

The main sources of plant auxin biosynthesis are young shoot tissues, primarily shoot apical meristem, leaf primordia, and young leaves, of which auxin is polarly transported from shoot to roots (Swarup and Bennett 2014). However, the local biosynthesis of auxin in roots is also necessary for root meristem maintenance and viability (Brumos, Robles et al. 2018). The polar auxin transport (PAT) is mediated by both auxin influx carriers, AUX/LAX (AUXIN-RESISTANT1–LIKES), and efflux carriers PINs (PIN-FORMED), generating the cell-to-cell movement of auxin (Swarup and Bhosale 2019, Han, Adamowski et al. 2021). The directional movement of auxin by PAT creates the auxin concentration gradient ranging from the maximum to the minimum which is required for determining the developmental pattern, developmental transition, directional growth movement, and meristematic function (Petricka, Winter et al. 2012, Habets and Offringa 2014, Di Mambro, De Ruvo et al. 2017, Brumos, Robles et al. 2018). The function of auxin transporters is required for the local auxin accumulation in the developing LRPs. As shown by previous studies, auxin transporter genes expressed in developing LRPs (Ni, Shen et al. 2014, Wang, Xuan et al. 2020) and factors

that cause changes in their expression affected the development of lateral roots (Wang, Xuan et al. 2020). The knock-down mutation of auxin influx carriers, OsAUX1 and OsAUX3, reduced lateral root number and LRPs, while the overexpression of the genes increased indicating their essential role in the lateral root initiation (Zhao, Ma et al. 2015, Wang, Qiao et al. 2019).

2.8 Brassinosteroid

Brassinosteroid is the plant steroid hormone that regulates a wide range of important developmental processes, including cell division, cell expansion, cell differentiation, vascular development, and pollen development (Li and He 2020, Oh, Honey et al. 2020), as observed in the brassinosteroid-related mutants that showed severe developmental defects such as stunt growth, dwarfism, and reduced fertility (Figure 14 and 15) (Clouse, Langford et al. 1996, Szekeres, Németh et al. 1996, Choe, Dilkes et al. 1998, Nakamura, Fujioka et al. 2006, Jiang, Huang et al. 2013, Li and He 2020). In rice, brassinosteroid controls many plant architectures, such as plant height, leaf growth angle, and grain size (Zhang, Bai et al. 2014, Hwang, Ryu et al. 2021). Either brassinosteroid-deficient or brassinosteroid-insensitive mutants had dwarfism, shorter cell size, non-elongated internodes, erect leaves, infertility, reduced grain size, and lower grain weight (Hong, Ueguchi-Tanaka et al. 2005, Nakamura, Fujioka et al. 2006, Bai, Zhang et al. 2007, Tong, Xiao et al. 2014).

Figure 14 Comparision between wildtype and brassinosteroid-related mutants at five-week-old *Arabidopsis*. Brassinosteroid-deficient mutants, *dwf5*, *det2*, *dwf1*, *dwf4*, and *cpd*, and brassinosteroid-insensitive mutant, *bri1*, respectively (Clouse 2011).

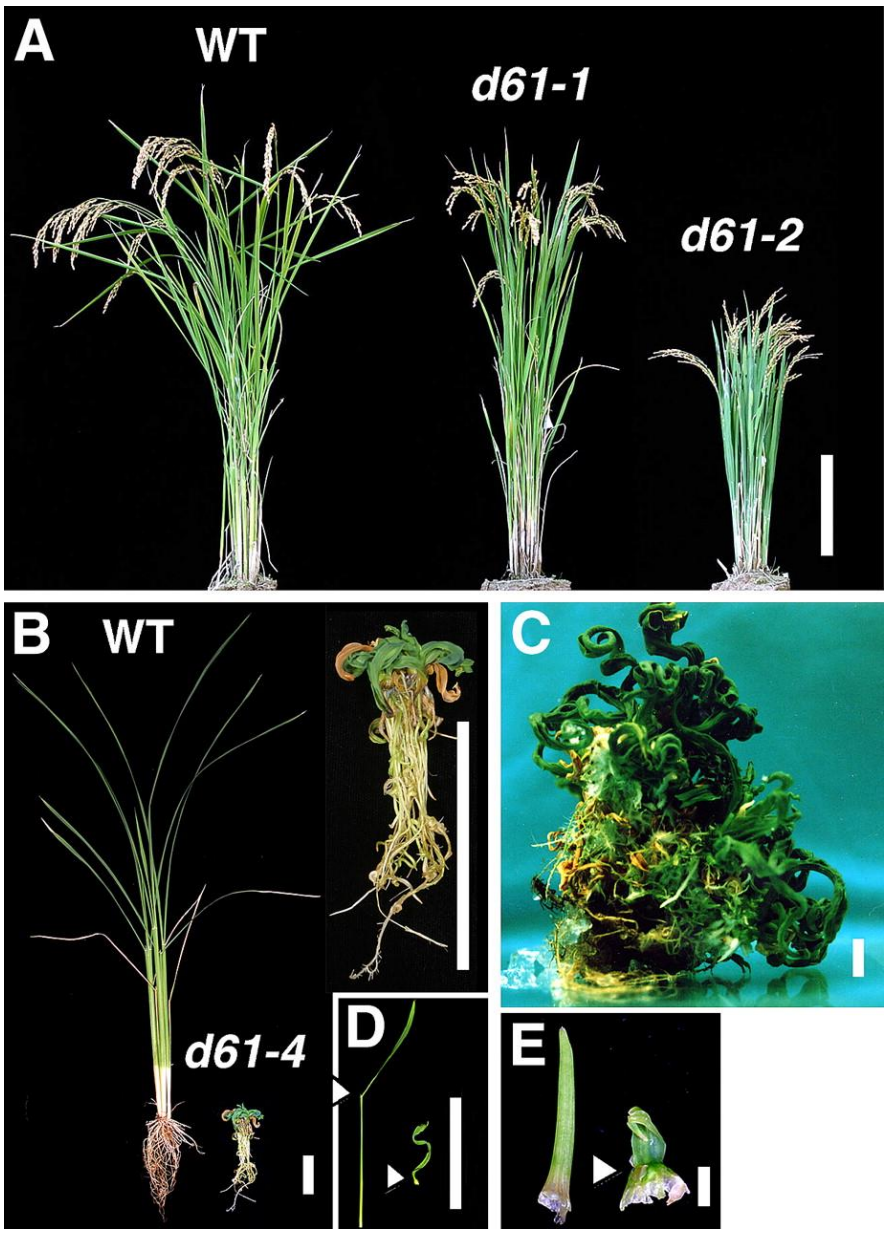

Figure 15 Comparision between wild type and brassinosteroid-insensitive mutant in rice. A). The shoot morphology of wild type and *d61* mutants with mild effects (*d61-1* and *d61-2*), which is the loss of function of *OsBRI1*. B). The whole plant morphology of wild type and *d61* mutants with severe effects (*d61-4*) at two months old. C). The close-up view of the shoot of *d61-4* at six months old. D). The leaf sheath of wild type (left) and *d61-4* (right). E). The first leaves of the wild type (left) and *d61-4* (right) (Nakamura, Fujioka et al. 2006). Arrows indicated the lamina joint in D-E). Scale bars in A). = 20 cm, B). = 5 cm, C). = 1 cm, D). = 5 cm, and E). = 1 mm.

Brassinosteroid is biosynthesized from campesterol with multistep pathways that the end product is castasterone which can be further converted to brassinolide, which is the most active brassinosteroid compound in dicot such as *Arabidopsis* (Castorina and Consonni 2020, Wei and Li 2020). However, previous studies suggested that castasterone is likely to be the most active brassinosteroid in cereal plants, including rice (Kim, Fujioka et al. 2008). The biosynthetic pathway is conserved between *Arabidopsis* and rice, while there is an alternative pathway found in rice (Figure 2.16) (Zhang, Bai et al. 2014, Castorina and Consonni 2020). Many brassinosteroid-biosynthetic enzymes are a member of CYP450 enzymes that catalyze the rate-limiting step(s) of brassinosteroid biosynthesis and are inhibited by propiconazole (Hartwig, Corvalan et al. 2012, Rozhon, Akter et al. 2019, Bajguz, Chmur et al. 2020). Brassinosteroid is perceived by its receptors localized on the cell membrane which is BRASSINOSTEROID INSENSITIVE1 (BRI1). The binding of brassinosteroid to BRI1 leads to the inhibition of BRASSINOSTEROID-INSENSITIVE 2 (BIN2) that phosphorylates BRASSINAZOLE-RESISTANT1 (BZR1) and bri1 EMS SUPPRESSOR1/BRASSINAZOLE RESISTANT1 (BES1/BZR2) when brassinosteroid is absent. Both BZR1 and BES1 are the downstream transcription factors of the brassinosteroid signalling that are functionally active in the unphosphorylated forms and regulate the expression of brassinosteroid-responsive genes (Figure 17 and 18) (Zhang, Bai et al. 2014, Wei and Li 2020, Hwang, Ryu et al. 2021). The transport of brassinosteroid appears to be short-distance and lacks the organ-to-organ movement (Symons and Reid 2004), underling the importance of the local homeostasis of brassinosteroid (Vukašinović, Wang et al. 2021). Recent research on the expression of brassinosteroid-biosynthetic genes in *Arabidopsis* roots indicated that not all cells have the complete brassinosteroid-biosynthetic steps. Thus, the completion requires the cell-to-cell movement of brassinosteroid intermediates (Vukašinović, Wang et al. 2021).

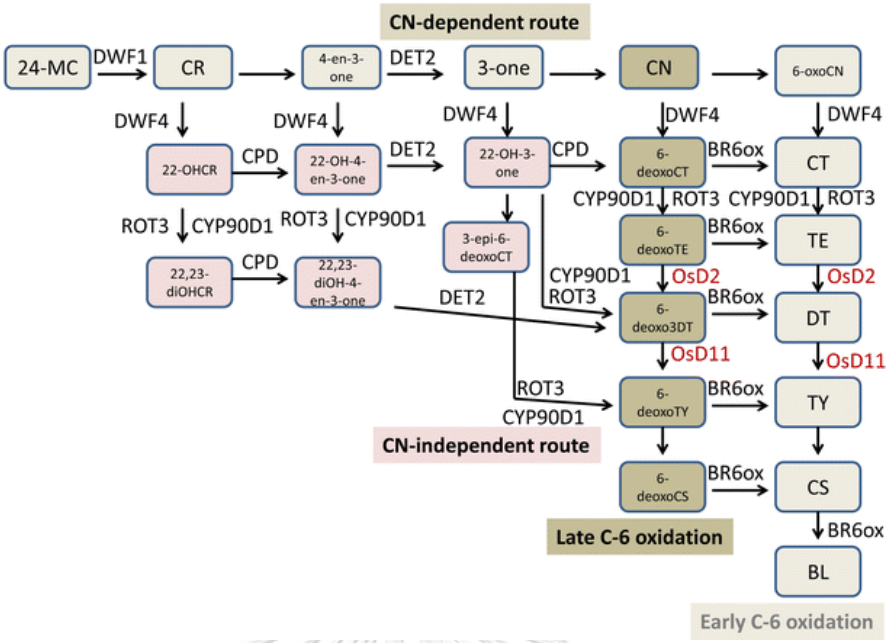

Figure 16 Brassinosteroid biosynthetic pathway in *Arabidopsis* and rice. Red colour represents the enzymes that have been found only in rice (Zhang, Bai et al. 2014). CR, campesterol; CN, campestanol; CT, cathasterone; TE, teasterone; DT, dehydroteasterone; TY, typhasterol; CS, castasterone; BL, brassinolide.

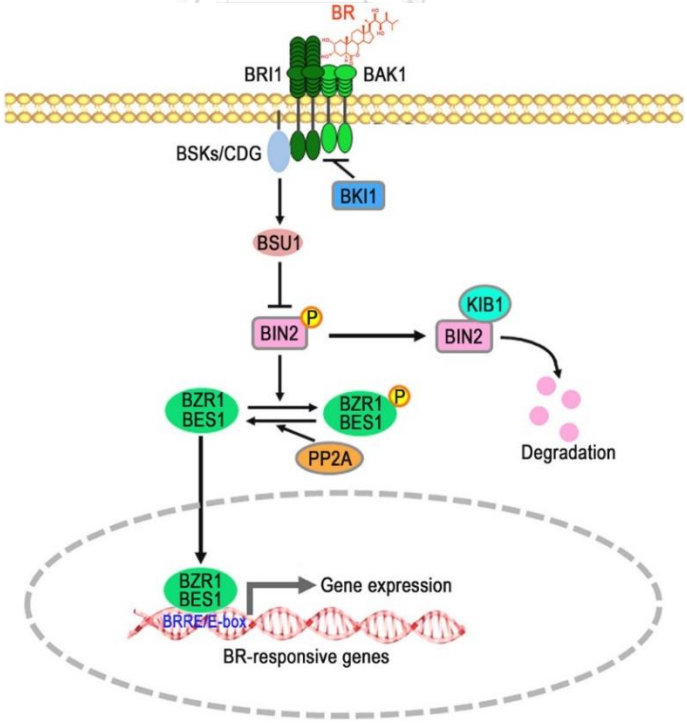

Figure 17 The signalling pathway of brassinosteroid (Li, Lu et al. 2018).

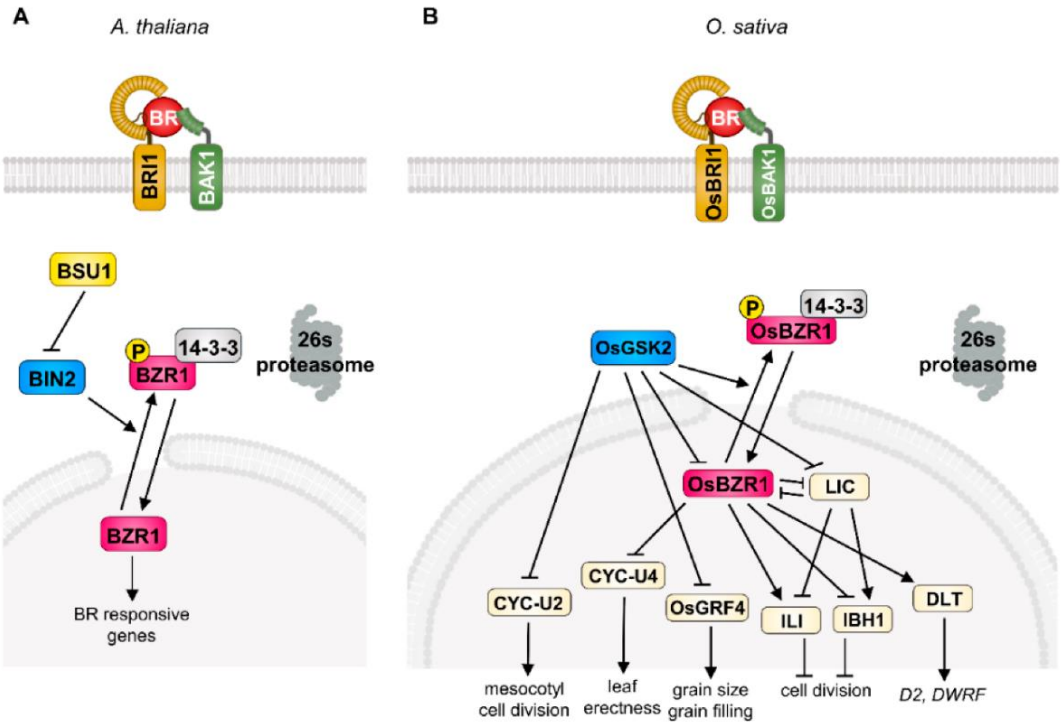

Figure 18 Comparison of the signalling pathway of brassinosteroid in *Arabidopsis* (A) and rice (B) with the brassinosteroid-responsive gene and traits that are regulated via the brassinosteroid signalling in rice. OsGSK2 is the homolog of Arabidopsis BIN2 in rice (Hwang, Ryu et al. 2021).

Brassinosteroid regulates root growth with concentration-dependent manners, which brassinosteroid showed stimulative effects at low concentrations, while root growth was inhibited at high concentrations (Clouse, Langford et al. 1996, Tong, Xiao et al. 2014, Vukašinović, Wang et al. 2021). In addition, a recent study demonstrated that different developmental zones in root tips required different concentrations of brassinosteroid for optimal growth and development (Vukašinović, Wang et al. 2021). Brassinosteroid also has an essential role in the regulation of lateral root development. Studies in *Arabidopsis* revealed that brassinosteroid positively regulated lateral root emergence at low concentrations by increasing the acropetal auxin transport, while high concentrations of brassinosteroid showed inhibitory effects (Bao, Shen et al. 2004, Gupta, Singh et al. 2015). Defects in the development of lateral roots were also observed in brassinosteroid-related mutants, both deficient and insensitive mutants in *Arabidopsis*, *dwf4-44* (Hou, Zhang et al. 2019), *bri1-6* (Gupta, Singh et al. 2015) and rice, *d61* mutants (Nakamura, Fujioka et al. 2006). The mutants developed fewer lateral root numbers with lower lateral root density compared to their wild type background (Nakamura, Fujioka et al. 2006, Gupta, Singh et al. 2015, Hou, Zhang et al. 2019). However, how brassinosteroid regulates the development of lateral roots in rice have not been well studied.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Tools and equipment

3.1.1.1 Digital camera

3.1.1.2 Computer

3.1.1.3 Borosilicate glass bottle

3.1.1.4 Stereo light microscope

3.1.1.5 Compound light microscope

3.1.1.6 Refrigerator

3.1.1.7 Laminar air-flow cabinet

3.1.1.8 Alcohol burner

3.1.1.9 Needle

3.1.1.10 Microscopic slides and cover slips

3.1.1.11 Autoclave

3.1.1.12 Forceps

3.1.1.13 Pipette tip

3.1.1.14 Automatic adjustable micropipette

3.1.1.15 Microwave

3.1.1.16 pH meter

3.1.1.17 Petri dish

3.1.1.18 Aluminum foil

3.1.1.19 Analytical Balance with 4 digits

3.1.1.20 Spatula

3.1.1.21 A flatbed scanner (EPSON Perfection V850 Pro, Japan)

3.1.2 Chemicals

3.1.2.1 NH_4NO_3

3.1.2.2 $NaH_2PO_42H_2O$

3.1.2.3 K_2SO_4

3.1.2.4 $CaCl_2$

3.1.2.5 $MgSO_4 7H_2O$

3.1.2.6 $MnCl_2 4H_2O$

3.1.2.7 $(NH_4)_6Mo_7O_{24} 4H_2O$

3.1.2.8 H_3BO_3

3.1.2.9 $ZnSO_4 7H_2O$

3.1.2.10 $CuSO_4 5H_2O$

3.1.2.11 Fe-EDTA

3.1.2.12 Citric acid (monohydrate)

3.1.2.13 NaOH

3.1.2.14 MES monohydrate

3.1.2.15 Ethanol

3.1.2.16 Gellan gum

3.1.2.17 Methylene blue

3.1.2.18 Acetic acid

3.1.2.19 Haiter commercial bleach

3.1.2.20 Sucrose

3.1.2.21 1-Naphthaleneacetic acid (NAA)

3.1.2.22 24-epicastasterone (Yuanye Biology, Shanghai, China)

3.1.2.23 Propiconazole (Syngenta, Shanghai, China)

3.1.2.24 Distilled water

3.1.2.25 Glycerol

3.1.2.26 Formaldehyde

3.1.3 Plant material

Seeds of Thai local rice, Puang Tawng (G.S. number 574), were obtained from Pathum Thani rice research center.

3.2 Methods

3.2.1 Growth condition

As illustrated in figure 19, dehusked seeds were surface sterilized by soaking with 70 % ethanol for 2 minutes, 40 % Haiter commercial bleach for 15 minutes, and then rinsed with sterilized dH_2O for 4-5 times. The sterilized seeds were germinated on sterile plates containing Yoshida's solution (Yoshida 1976) with 1 % sucrose, 0.55 g/L MES, and 0.25 % gellan gum under dark condition at room temperature for two days. Uniform seedlings of which primary root length was approximately 1 cm were selected and transferred to a glass tube (one plant per tube) containing the media as described below.

3.2.1.1 Control treatment: Yoshida's solution (Yoshida 1976) with 1 % sucrose, 0.55 g/L MES, and 0.25 % gellan gum. Yoshida's solution was prepared from the stock solution (1.25 ml of each stock solution per liter of media) as described in Appendix 4.

3.2.1.2 Propiconazole treatments: The same media as control supplemented with propiconazole at the concentration of 0.1, 1, 5, 10, and 20 µM.

3.2.1.3 1-Naphthaleneacetic acid (NAA) treatments: The same media as control supplemented with NAA at the concentration of 20, 50, and 100 nM.

3.2.1.4 24-epicastasterone treatments: The same media as control supplemented with 24-epicastasterone at the concentration of 0.1, 1, 10, and 100 nM.

3.2.1.5 1-Naphthaleneacetic acid (NAA) cotreated with propiconazole: The same media as 3.2.1.3 cotreated with 5 µM propiconazole.

3.2.1.6 24-epicastasterone cotreated with propiconazole: The same media as 3.2.1.4 cotreated with 5 µM propiconazole.

All plants were grown in a phytotron room (30 ^{O}C and 12-hour-light-dark cycle) for another 5 or 8 days.

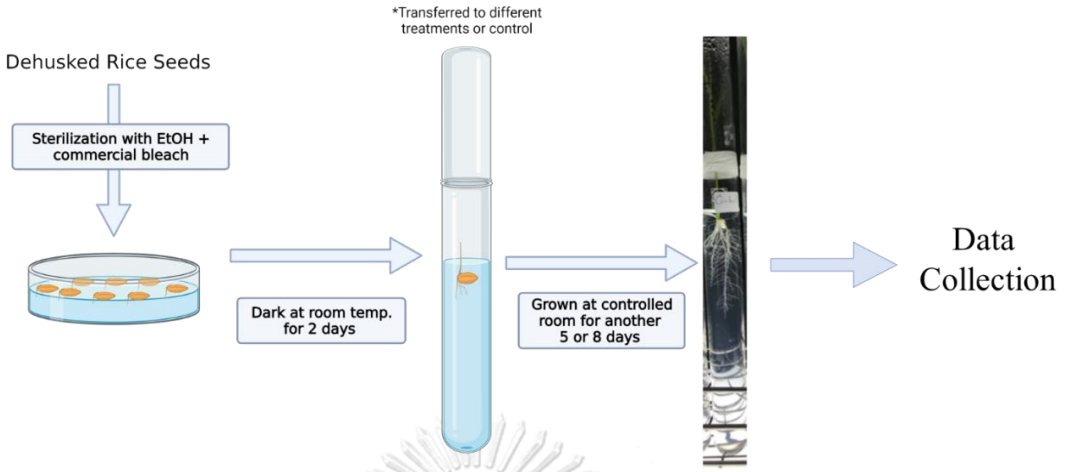

Figure 19 Diagram of the method for plant growth. Created with BioRender.com

3.2.2 Measurement of shoot and root growth parameters

After 5 or 8 days of the treatments, growth parameters were collected as below. On the day of data collection, all roots were fixed in either 70% ethanol or FAA solution (Formaldehyde Alcohol Acetic Acid, 10%:50%:5% + 35% water) if roots were then subjected to counting lateral root primordia density.

3.2.2.1 Shoot fresh weight (g)

3.2.2.2 Shoot dry weight (g)

3.2.2.3 Root fresh weight (g)

3.2.2.4 Plant height (cm)

3.2.2.5 Primary root length (cm)

Roots were scanned by Epson Perfection V700 Photo scanner. Primary root length was measured by the scanned photo by using ImageJ software version Fiji (Schindelin et al., 2012).

3.2.2.6 Total root length (cm), average root diameter (mm) and root surface area (mm^2)

Total root length, average root diameter and root surface area were analyzed by RhizoVision Explorer (Seethepalli, Dhakal et al. 2021), which is open-source software for root image analysis.

3.2.2.7 Lateral root density (lateral root number/ primary root length)

Lateral root density was calculated by the number of lateral roots on the primary root divided by the primary root length. Emerged lateral roots were defined as the lateral roots that emerged out of the root epidermis, while those that were still inside the root were defined as unemerged lateral roots. Total lateral root density was calculated from the number of both emerged lateral roots and unemerged lateral roots that divided by the primary root length. The number of lateral roots was manually counted under a stereomicroscope. Lateral root primordia were observed and counted as described in the previous studies (Chen, Shi et al. 2013, Ni, Shen et al. 2014). Primary roots were fixed in the FAA solution for several days. Then, the roots were divided into each 1-cm long section from the root tip, dissected the cortex out, and stained with methylene blue. The dissection of the root cortex and counting the number of lateral root primordia were performed under a stereomicroscope.

3.2.2.8 The developmental stages of lateral root primordia

For the identification of developmental stages of the unemerged LRPs in the root maturation zone, root sections were incubated overnight with a basic solution (7% NaOH in 60% ethanol) and then mounted in a solution (50% glycerol in 10% ethanol) (Xu, Zou et al. 2019) on the slide before imaging with a light compound microscope. Unemerged LRPs

3.2.3 Statistical Analysis

All experiments used completely randomized design (CRD). The results in 4.1 and 4.3 were analyzed by ANOVA, and the results in 4.2 were analyzed by Student’s T-Test . All statistical analyses were performed by using SPSS software version 22 (IBM, New York, USA). Means were calculated from 15 replicates for the results in 4.1, 14-15 replicates for the results in 4.3, and 20 replicates for the results in 4.2. Statistical differences of the means in 4.1 and 4.3 were analyzed by Tukey's HSD test for the parameters that equal variances were assumed and Dunnett's T3 test for the parameters that equal variances were not assumed according to Levene's test for equality of variances (Test of homogeneity of variances).

CHAPTER IV
RESULTS

4.1 Effects of propiconazole on plant growth in rice seedlings.

To investigate how propiconazole affects plant growth, the varying concentrations of propiconazole at the concentration of 0.1, 1, 5, 10, and 20 μM were used to treat rice seedlings. Propiconazole showed noticeable inhibitory effects at the concentration as low as 1 μM that plant height and shoot fresh weight appeared to be significantly inhibited (figure 20 and 22). At the higher concentration, propiconazole inhibited plant growth more severely. Plant height of the propiconazole-treated plants at the concentration of 10 and 20 μM were approximately half of the untreated plants. In addition, leaf morphology was also obviously affected. Leaves of the propiconazole-treated plants were shorter, malformed, and twisted, similar to phenotypes observed in brassinosteroid-insensitive and -deficient mutants (figure 20) (Hong, Ueguchi-Tanaka et al. 2005, Nakamura, Fujioka et al. 2006).

High concentrations of propiconazole also negatively affected root growth, which the primary root length of the propiconazole-treated plants was dramatically shorter and root fresh weight was significantly reduced when seedlings were treated with 20 μM propiconazole (figure 21 and 23). In addition, total root length and root surface area of the whole root system were significantly reduced at the concentration as low as 1 μM and propiconazole showed more severe effects when plants were treated with higher concentrations, which total root length and root surface area of the seedlings treated with 20 μM propiconazole were only about 16% and 40% compared to those of the untreated plants, respectively (figure 21 and 23). Moreover, roots of propiconazole-treated plants with 1 μM and higher were noticeably thicker than the untreated roots due to their higher average root diameter (figure 21 and 23). Another inhibitory effect of propiconazole was observed on lateral root development. It was severely inhibited by propiconazole at the concentration as low as 1 μM due to the significant decrease in the density of emerged lateral roots of the primary root, which was about 70% compared to the untreated plants (figure 21 and figure 23).

From the results, 0.1 μM propiconazole did not significantly affect all the traits observed in this experiment neither positively nor negatively. 5 μM propiconazole was selected for the following experiments because it showed obvious inhibitory effects on rice seedling growth and did not cause too severe developmental defects as observed in the plants treated with 20 μM propiconazole (figure 20-23).

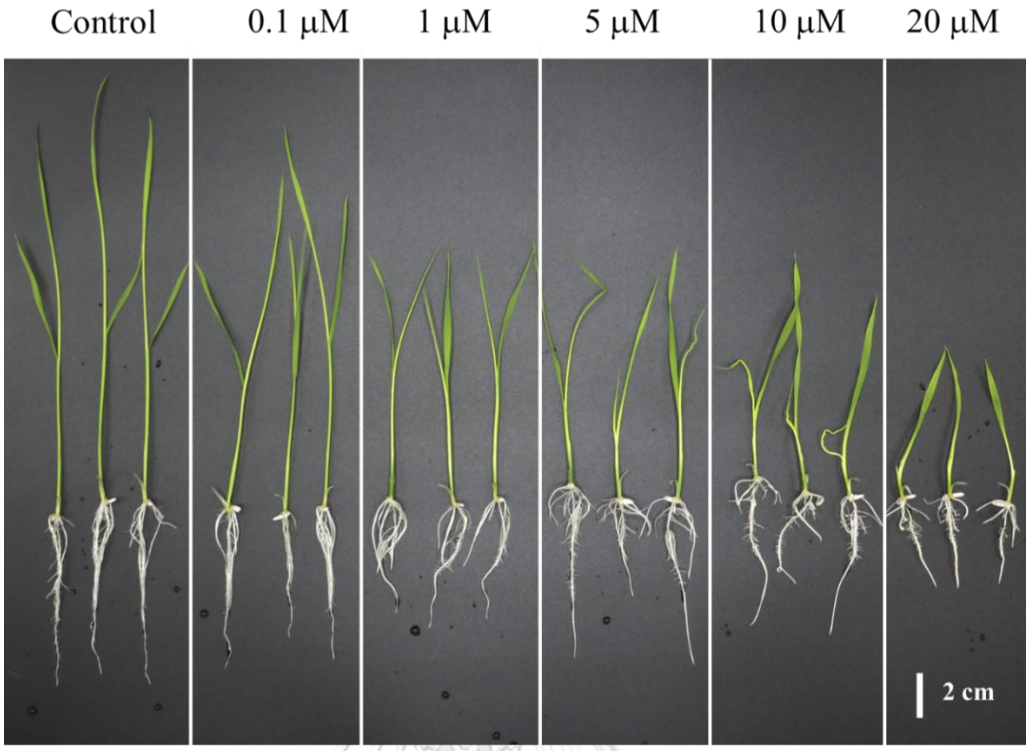

Figure 20 Rice seedlings treated with the varying concentrations of propiconazole for five days

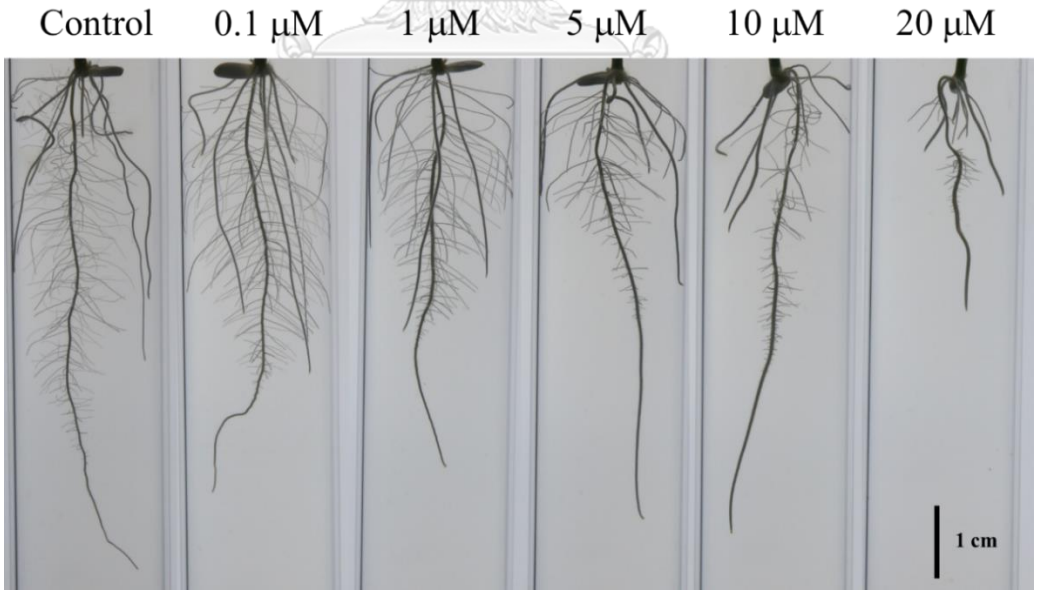

Figure 21 Roots of rice seedlings treated with the varying concentrations of propiconazole for five days.

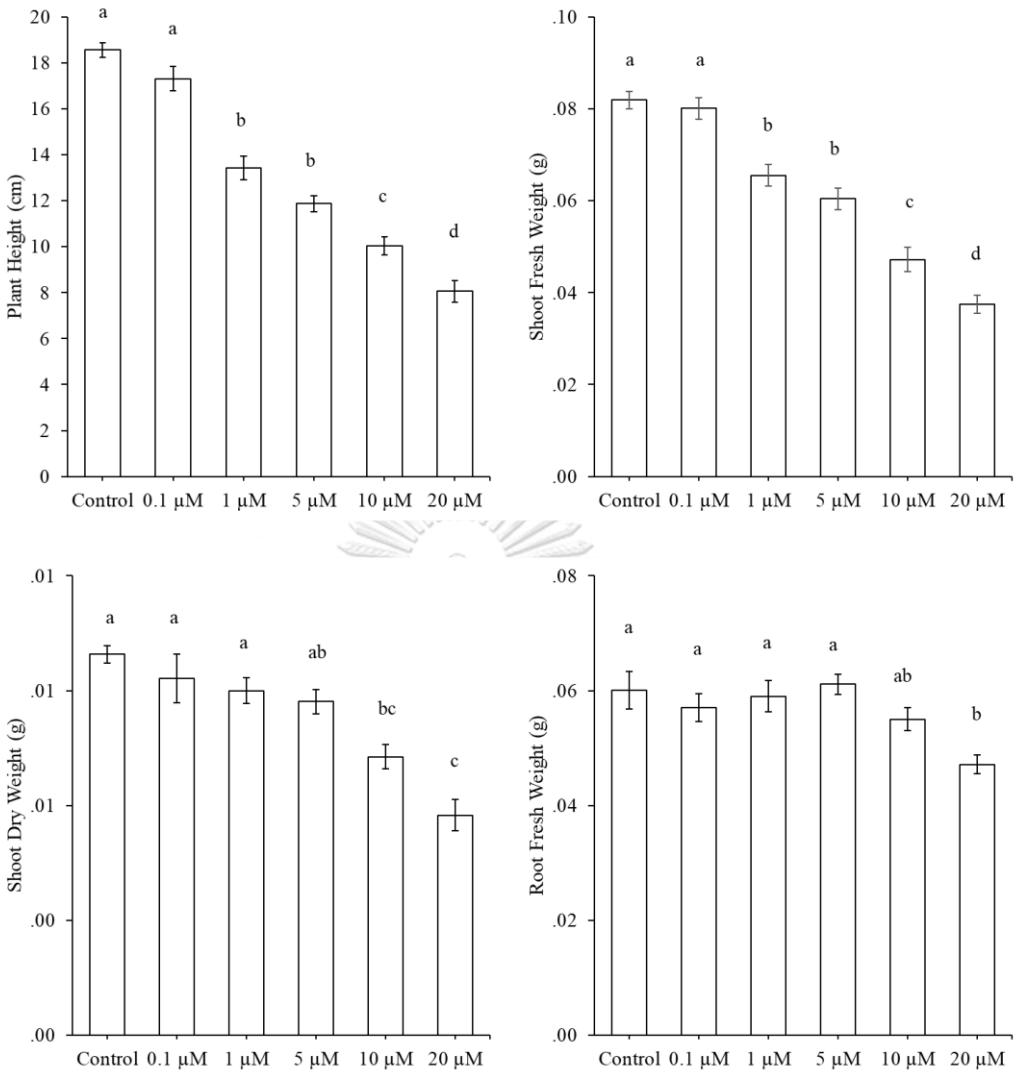

Figure 22 Plant growth traits of the rice seedlings treated with the varying concentrations of propiconazole for five days. Error bar at each point represents ± SD. Significant differences of the mean were analyzed by Tukey's HSD test ($P < 0.05$) and indicated by different letters above the bar graph.

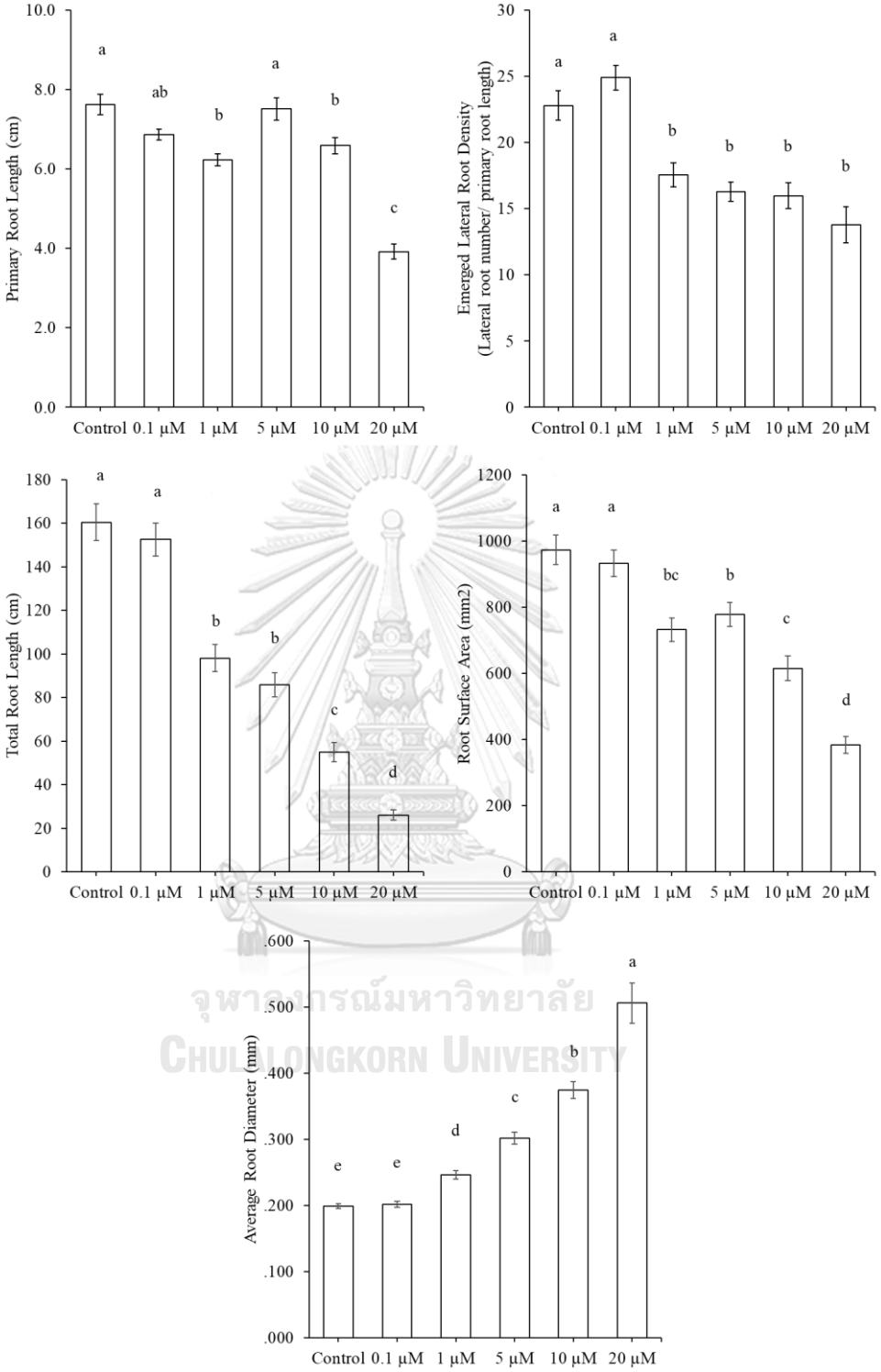

Figure 23 Root traits of the rice seedlings treated with the varying concentrations of propiconazole for five days. Error bar at each point represents ± SE. Significant differences of the mean were indicated by different letters above the bar graph, which were analyzed by Tukey's HSD test ($P < 0.05$) for primary root length, emerged lateral root density and root surface area and Dunnett's T3 test ($P < 0.05$) for total root length and average root diameter.

4.2 Inhibition of lateral root development by propiconazole.

The development of lateral roots begins with the priming processes and fully develops when lateral roots emerge out of their parent root (Lavenus, Goh et al. 2013, Du and Scheres 2017). This study separated the development of lateral roots into two main steps which are initiation and emergence. Emerged lateral root density in the seedlings treated with 5 μM propiconazole was dramatically reduced (figure 24 and 25). This suggested that propiconazole possibly inhibited either the initiation or the emergence of LRP. To identify the developmental process that was affected by propiconazole, the number of LPR inside the primary root was observed and counted. Total lateral root density including both emerged lateral roots and LRPs, which are unemerged lateral roots, decreased approximately 10% in the propiconazole-treated roots compared to the control treatment (figure 25). However, the proportion of LRPs in the propiconazole-treated roots was account for about 50% of the total lateral root density, while it was less than 10% in the control treatment. This finding suggested that propiconazole negatively affected the development of lateral roots likely by inhibiting the emergence of LRPs, while the inhibitory effect on the initiation was less obvious due to the slight decrease in the total lateral root density in the propiconazole-treated roots (figure 25).

To further support this observation, sections from the primary root were closely investigated. As shown in figure 26, emerged lateral roots were found in the 0-1 cm section from the root tip of the control plants, while emerged lateral roots were rarely found on the section of the propiconazole-treated plants. In the older region from the 1-2 cm section from the root tip, the lateral roots of the control root section all maturely developed and emerged out of their parent root with distinct elongation of newly emerged lateral roots. In contrast, many LRPs were found within the 1-2 cm section of the propiconazole-treated roots, and few lateral roots were just about to emerge. Interestingly, LRPs were still found in the root section above 5 cm from the root tip of the propiconazole-treated roots as shown in figure 27. To identify the defective stage of the unemerged LRPs, the LRPs from the 3-4 cm section and the 4-5 cm section from the root tip of the propiconazole-treated roots were further examined. As shown in figure 28 and 29, the LRPs had a mushroom-like or dome shape, and the structure of LRPs resembled the mature root tip. This finding further suggested that propiconazole inhibited the emergence of LRPs. Furthermore, the lateral root length of the propiconazole-treated plants was evidently shorter than those of the control treatment, additionally suggesting that propiconazole also inhibited lateral root elongation. Taken together, it can be concluded that propiconazole negatively affected the emergence of LRP by inhibiting the elongation of lateral roots.

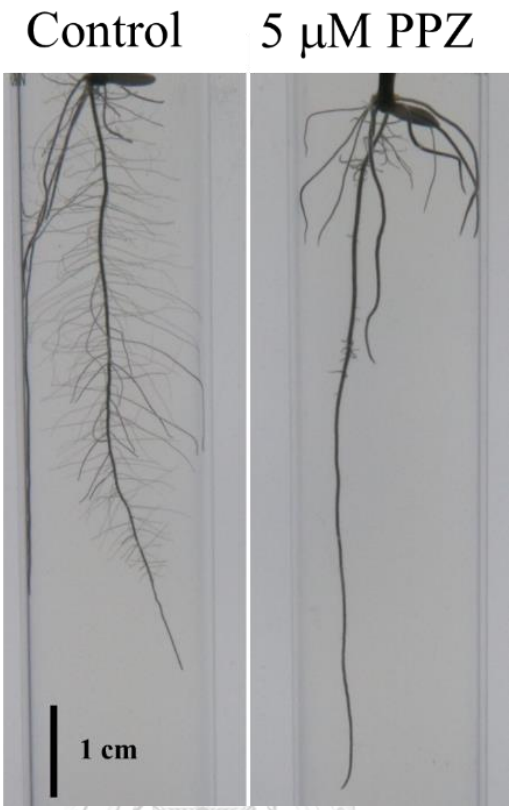

Figure 24 Roots of rice seedlings without (left) and with 5 μM propiconazole (PPZ) treatment (right) for five days.

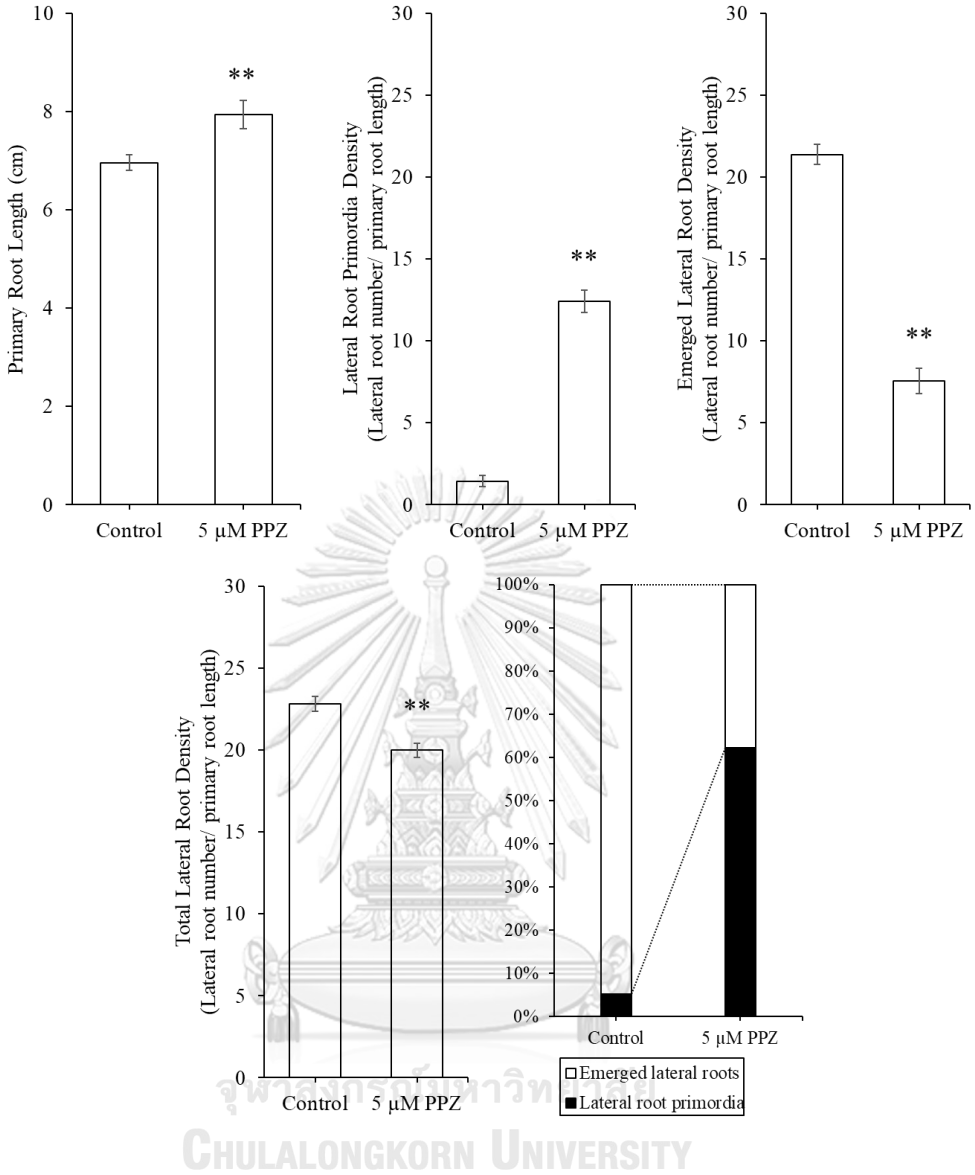

Figure 25 Quantification of root traits of the rice seedlings with and without 5 µM propiconazole treatment for five days, which are primary root length, lateral root primordia density, emerged lateral root density, total lateral root density, and the proportion between lateral root primordia and emerged lateral roots shown in percentage. Error bar at each point represents ± SE. ** above the bar graphs indicated the significant differences of the means analyzed by Student's T-Test ($P < 0.01$).

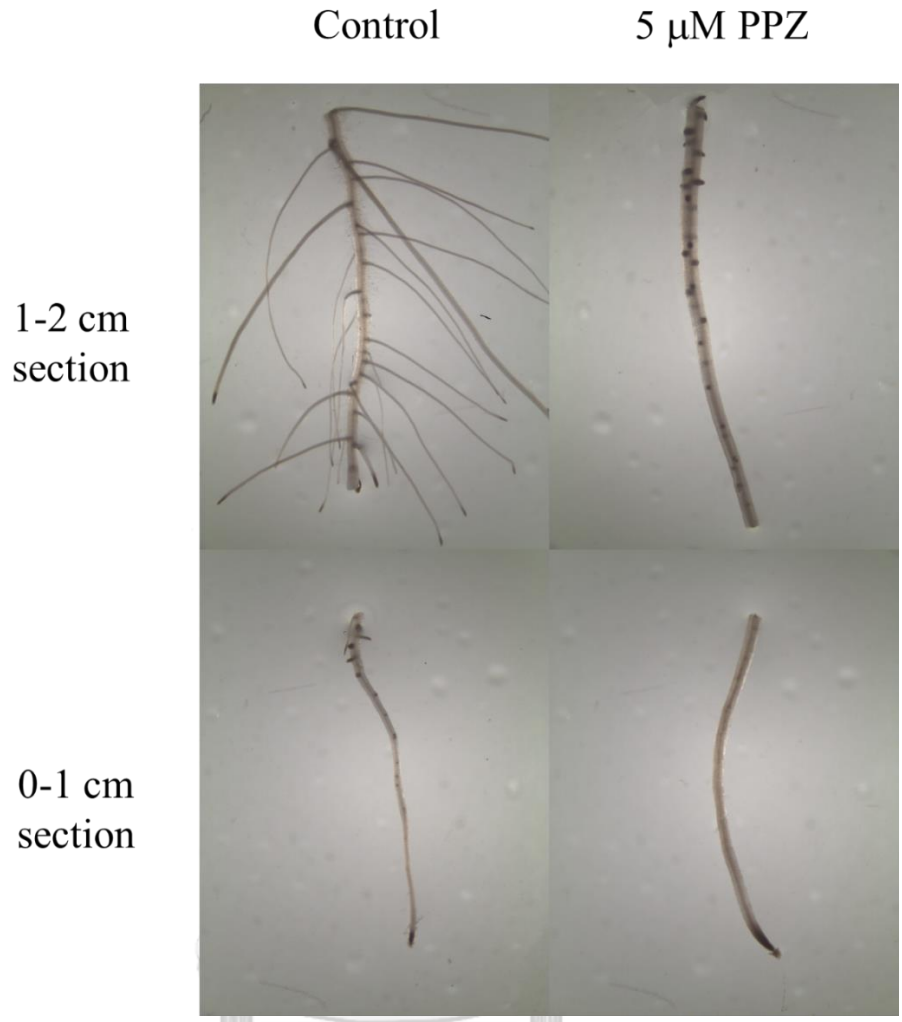

Figure 26 Sections from 0-1 and 1-2 cm from the root tip of the primary root of rice seedlings without (left) and with 5 µM propiconazole treatment (right) for five days.

Figure 27 Section from above 5 cm from the root tip of the primary root of rice seedlings treated with 5 µM propiconazole for five days. Arrows indicate lateral root primordia or unemerged lateral roots.

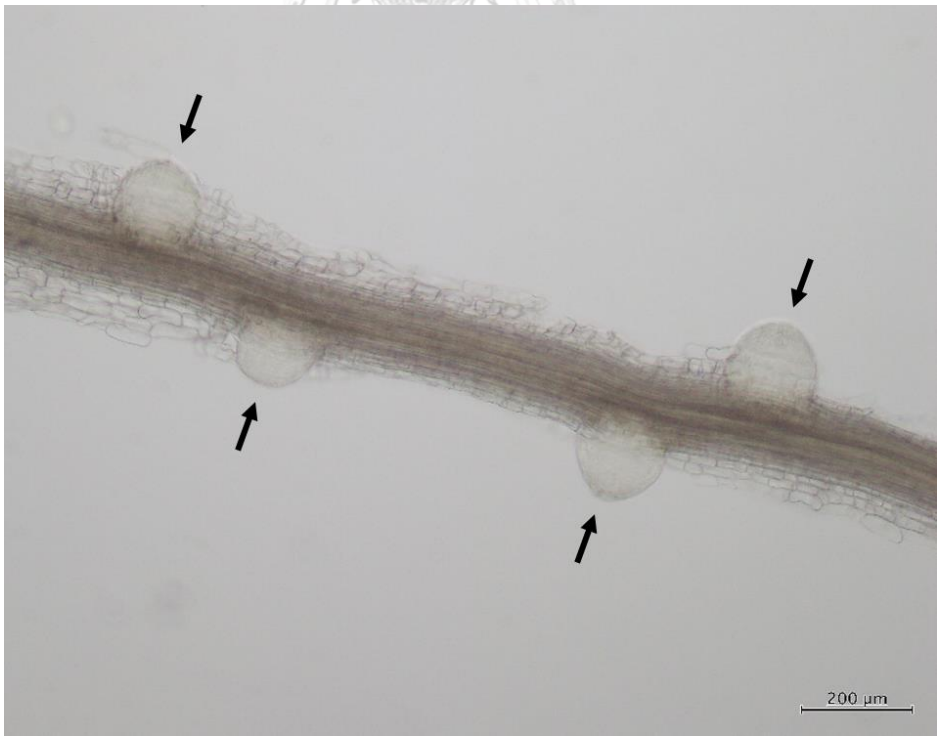

Figure 28 Lateral root primordia or unemerged lateral roots with the removal of the cortex of the section from 3-4 cm from the root tip of the primary root of rice seedlings treated with 5 µM propiconazole for five days. Arrows indicate lateral root primordia or unemerged lateral roots.

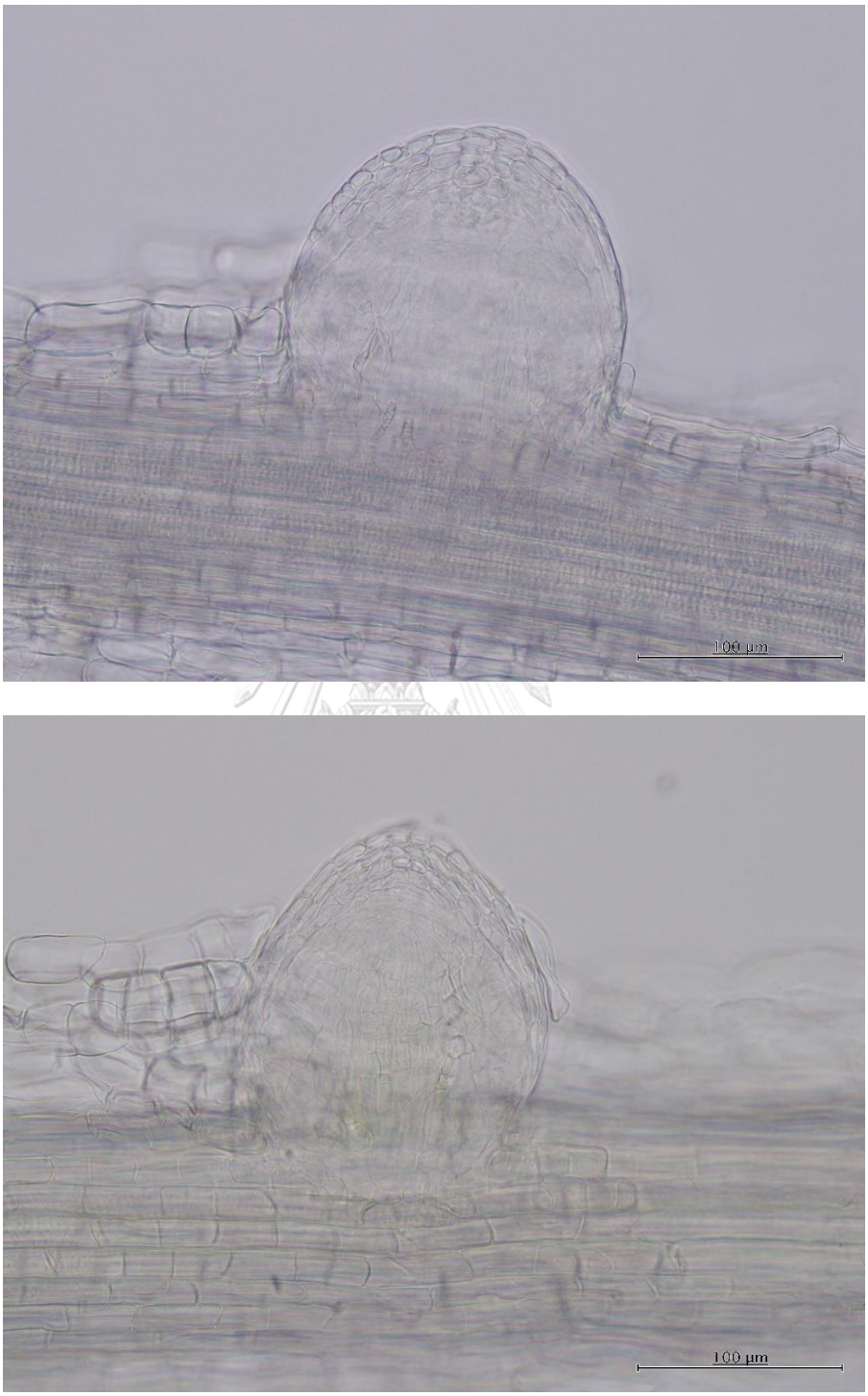

Figure 29 Close up of lateral root primordia or unemerged lateral roots of the section from 3-4 cm (above) and 4-5 cm (below) with the removal of the cortex from the root tip of the primary root of rice seedlings treated with 5 µM propiconazole for five days.

4.3 The inhibitory effects of propiconazole on lateral root development were alleviated by exogenous auxin.

Auxin is known as the key plant hormone that regulates lateral root development. Therefore, the abnormal development of lateral roots observed in the propiconazole-treated plants is possibly caused by the disturbance of auxin action. To test this, the propiconazole-treated plants were grown in the presence of 1-naphthaleneacetic acid (NAA), a synthetic auxin. NAA treatments showed stimulatory effects on lateral root development, while primary root growth was inhibited by high concentrations of NAA (figure 30 and 31), which is consistent with previous studies (Sreevidya, Hernandez-Oane et al. 2010, Yoshikawa, Ito et al. 2014). Roots treated with NAA had significantly higher emerged lateral root density than the untreated roots with no propiconazole and NAA as shown in figure 30 and 31. 20 nM NAA was able to rescue the lateral root density to approximately the same level as the untreated roots. Interestingly, 50 nM and 100 nM NAA further increased emerged lateral root density in the propiconazole-treated roots to the level that was significantly higher than that of the untreated roots and the same level as the roots treated with only NAA (figure 31). Taken together, NAA cotreatment with propiconazole was able to alleviate propiconazole-induced inhibition of lateral root development, suggesting that propiconazole possibly caused the defects in auxin-mediated mechanisms required for the normal development of lateral roots.

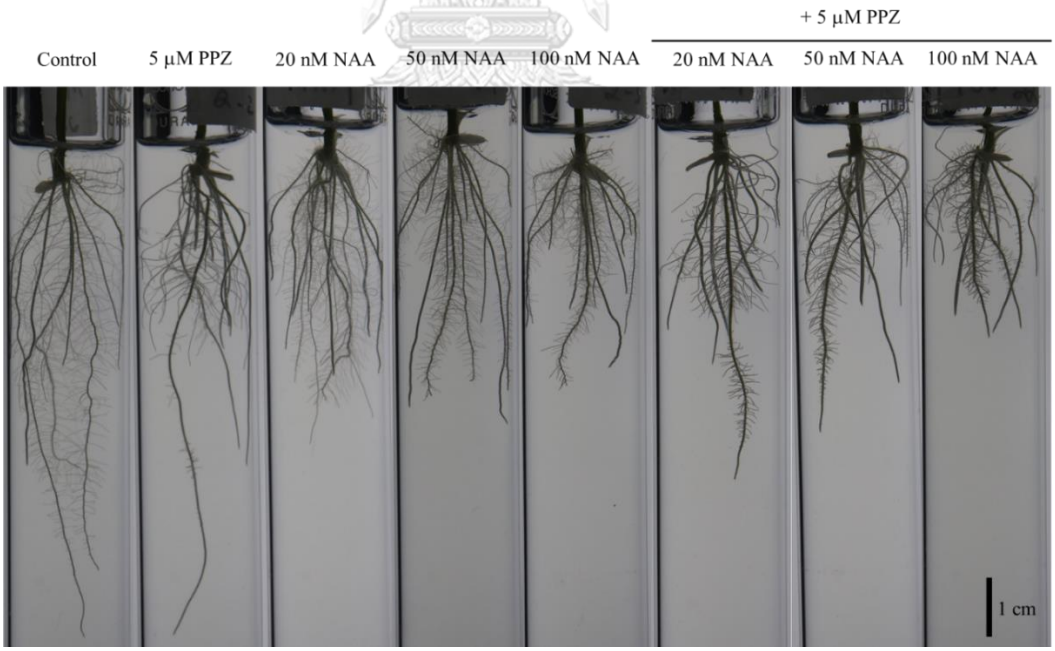

Figure 30 Response of roots treated with auxin (NAA) with/without 5 μM propiconazole. Roots of the rice seedlings treated with the varying concentrations of NAA with/without 5 μM propiconazole (PPZ) for 8 days.

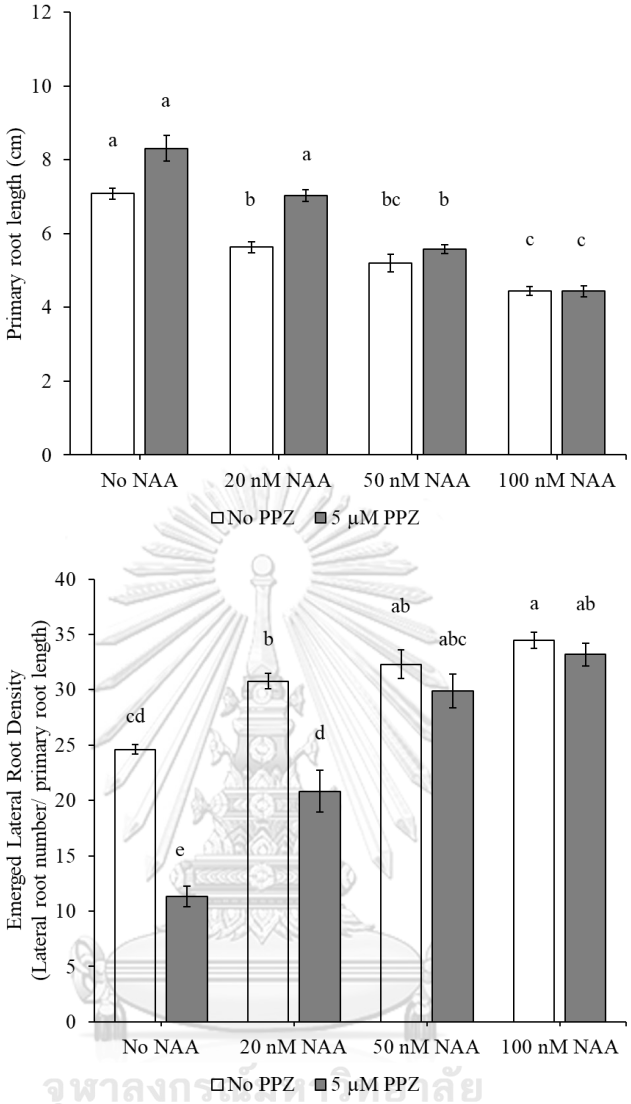

Figure 31 Quantification of primary root length and emerged lateral root density of the rice seedlings treated with NAA with/without 5 µM propiconazole for 8 days. Error bar at each point represents ± SE. Significant differences of the means were indicated by different letters above the bar graph, which were analyzed by Dunnett's T3 test ($P < 0.05$) for primary root length, emerged lateral root density and root surface area and Dunnett's T3 test for total root length and average root diameter.

4.4 Effects of 24-epicastasterone on the propiconazole-treated plants

Propiconazole is a specific inhibitor of the brassinosteroid biosynthesis (Hartwig, Corvalan et al. 2012, Oh, Matsumoto et al. 2016, Rozhon, Akter et al. 2019). Therefore, how brassinosteroid affects the growth of propiconazole-treated plants was investigated in this study. Although propiconazole negatively affected shoot growth, which caused shorter plant height in all propiconazole treatments both

with and without 24-epicastasterone (ECS) (figure 32), its inhibitory effects on lateral root development were not as noticeable as the other experiments in 4.1-4.3. Brassinosteroid is known to regulate root growth in the concentration-dependent manners (Bao, Shen et al. 2004, Tong, Xiao et al. 2014, Hou, Zhang et al. 2019, Jiao, Wang et al. 2019) and different tissues require different concentrations of brassinosteroid for their optimal growth (Vukašinović, Wang et al. 2021), indicating that factors affecting the biosynthesis or the endogenous contents of brassinosteroid thus have influences on root growth. In this experiment, there were at least two factors that could affect the endogenous contents of brassinosteroid, which are propiconazole (Hartwig, Corvalan et al. 2012) and NH_4^+ (Jiao, Wang et al. 2019) from NH_4NO_3 used in all treatment media. Plants can uptake various forms of nitrogen including NH_4^+ and NO_3^-, which are the major forms of inorganic nitrogen of which plant uptake capability varies upon the changing root media condition (Raj, Jhariya et al. 2021). A recent research demonstrated that high concentrations of NH_4^+ induce root growth inhibition by promoting the biosynthesis of brassinosteroid, leading to higher endogenous brassinosteroid contents to the levels that inhibit root growth (Jiao, Wang et al. 2019). This suggested that the variation of NH_4^+ concentrations might affect the endogenous brassinosteroid status, which possibly caused the observed variation in the effects of propiconazole treatments. Despite this observed variation, three independent experiments conducted in 4.1-4.3 showed consistency in the inhibitory effects on lateral root development.

High concentration of ECS (≥10 nM) evidently inhibited the primary root growth, resulting in the shorter primary root length as shown in figure 33, consistent with known effects of brassinosteroid that were previously reported (Clouse, Langford et al. 1996, Tong, Xiao et al. 2014, Hou, Zhang et al. 2019, Jiao, Wang et al. 2019). Considering under the assumption that the inhibitory effects of propiconazole are the same as what was observed in the experiment 4.1-4.3, high concentration (≥50 nM) of ECS was likely to partly rescue the lateral root density in the propiconazole treated plants.

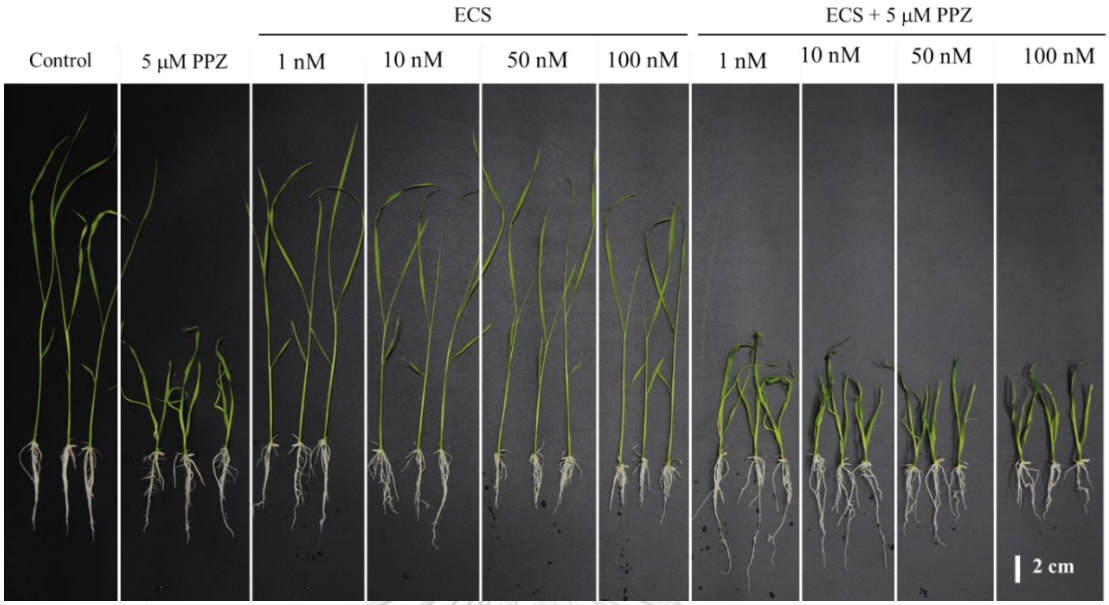

Figure 32 Response of rice seedling treated with the varying concentrations of brassinosteroid (24-epicastasterone, ECS) with/without 5 μM propiconazole (PPZ) for 8 days.

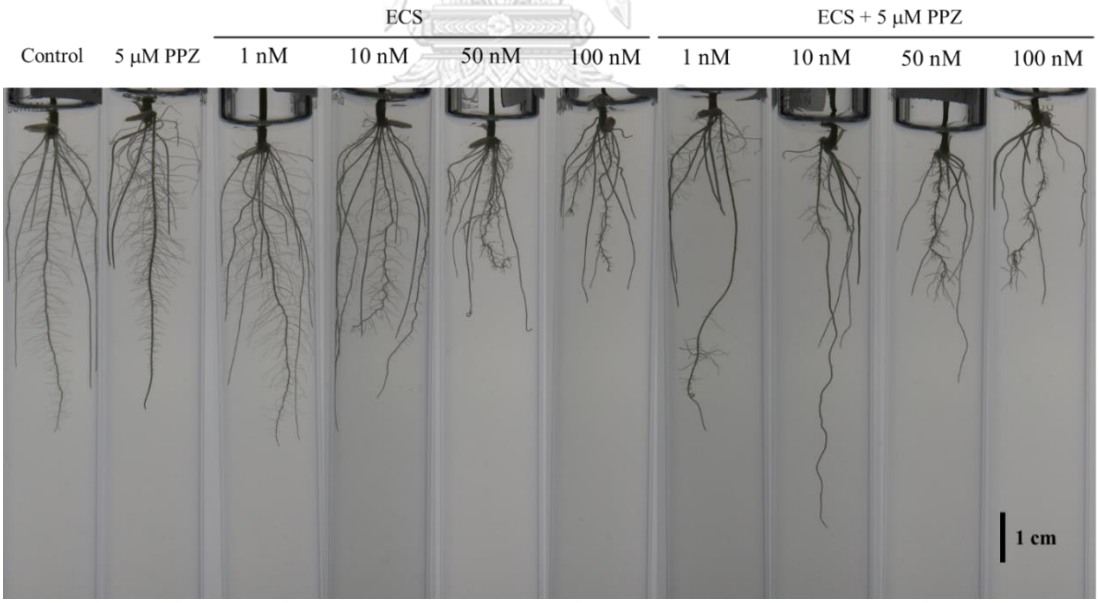

Figure 33 Response of roots treated with the varying concentrations of brassinosteroid (24-epicastasterone, ECS) with/without 5 μM propiconazole (PPZ) for 8 days.

CHAPTER V
Discussion

5.1 Propiconazole and the potential contamination problems.

Propiconazole negatively affected rice seedling growth as observed in the propiconazole-treated plants that showed retarded growth in both shoot and roots with obvious shoot dwarfism, shorter primary root length, lower total root length, lower root surface area, and lower lateral root density. Propiconazole is widely used as a fungicide to minimize crop damage from fungal diseases, and it is highly persistent in the soil after application because of its long haft-life time (Kim, Beaudette et al. 2002, Kim, Shim et al. 2003, Pan, Cheng et al. 2018). Moreover, there are pieces of evidence indicating that the accumulation of propiconazole in agricultural fields was detectable (Braun, Sebesvari et al. 2018, Tao, Jia et al. 2021). Therefore, the constant use of this fungicide could be vulnerable for the organisms in the surrounding environment and even the growing crop plants if the concentration of the contaminant exceeds its threshold to cause adverse effects (Bradley D, Carol A et al. 2003).

5.2 Propiconozole likely inhibits lateral root emergence.

Emerged lateral root density of the primary root was decreased in the propiconazole-treated rice seedlings, indicating the inhibitory effects on lateral root development. Further investigations indicated propiconazole may inhibit the emergence of developing LRPs and lateral root elongation that led to lower emerged lateral root density in the propiconazole-treated plants, supported by the following findings. Total lateral root density was only about 10% lower than the untreated roots. However, the emerged lateral root density was dramatically less than that of the untreated roots, suggesting that the initiation of the formation of LRPs was not obviously affected by the propiconazole treatments. When the proportion between emerged lateral roots and LRPs were analyzed, the proportion of LRPs that did not emerge out of their parent roots was about half of the total lateral root density. In addition, unemerged LRPs were found in the region that lateral roots maturely developed as observed in the untreated plants, for instance, above 3-4, 4-5, and even above 5 cm sections from the root tips. Moreover, those unemerged LRPs in the mentioned root sections were found to have a mushroom-like or dome shape and the structure resembling the mature root tips, indicating that the unemerged LRPs were mature (Ni, Shen et al. 2014). Furthermore, emerged lateral roots of the propiconazole were shorter than those of the untreated plants. Therefore, it was most likely that the emergence and elongation of lateral roots were inhibited by propiconazole.

5.3 Propiconazole interferes with auxin-mediated mechanisms that regulate lateral root development.

Auxin is known as the master regulator of lateral root development. Therefore, propiconazole possibly negatively affected auxin-mediated mechanisms. NAA treatments were able to rescue emerged lateral root density to those of the untreated

plants (figure 31), indicating that auxin-mediated mechanisms were affected. This could be the signalling, biosynthesis, or transported because changes in these factors are able to cause both positive and negative effects on lateral root development as reported in previous studies (Yamamoto, Kamiya et al. 2007, Jun, Gaohang et al. 2011, Zhu, Liu et al. 2012, Zhao, Ma et al. 2015, Zhang, Li et al. 2018, Wang, Qiao et al. 2019). Loss of function of auxin biosynthesis gene *OsTAA1* in rice led to the reduction of the IAA content in roots by half of that of its corresponding wild type. *Ostaa1* mutants had longer primary root length and very few or even no emerged lateral roots (Yoshikawa, Ito et al. 2014, Zhang, Li et al. 2018). The treatment of *p*-phenoxyphenyl boronic acid, a specific inhibitor of auxin biosynthesis that inhibits OsYUCCA activity, to rice seedlings resulted in longer primary root length and lower lateral root density at a low concentration, while a high concentration of the inhibitor inhibited both primary and lateral root development (Watanabe, Shigihara et al. 2021). Changes in the shoot-to-root auxin transport also affect the development of lateral roots. A previous study has reported that the shoot-to-root auxin transport was reduced under the treatment of strigolactones and nutrient-limiting conditions, resulting in lower auxin levels in roots, leading to longer primary root length and decreased lateral root density (Sun, Tao et al. 2014). The phenotypes were similar to what was observed in the propiconazole treated roots at moderate concentrations, such as 5 µM (figure 23, 25 and 31). The primary root length of the roots treated with 5 µM propiconazole was not shorter than the untreated plants but showed obvious reduction of emerged lateral root density (figure 23, 25 and 31). Therefore, propiconazole may affect endogenous auxin levels in roots, leading to the inhibitory effects on lateral root development. At the early stages of seedling development, lateral root emergence requires shoot-derived auxin. However, the requirement is reduced when seedlings become older and then dependent on local auxin biosynthesis instead (Bhalerao, Eklöf et al. 2002). In addition, a recent study demonstrated that local auxin biosynthesis in roots is required for maintaining root meristematic function in both primary roots and emerged lateral root in *Arabidopsis* (Brumos, Robles et al. 2018). Therefore, propiconazole might somehow affect local auxin biosynthesis in roots, leading to the inhibition of lateral root development.

5.4 Defects in lateral root development are possibly due to the deficiency of brassinosteroid induced by propiconazole.

Severe developmental defects were observed in the brassinosteroid-deficient mutants caused by the loss of function of brassinosteroid biosynthesis genes (Szekeres, Németh et al. 1996, Hong, Ueguchi-Tanaka et al. 2005, Vukašinović, Wang et al. 2021), and propiconazole specifically inhibits the biosynthesis of brassinosteroid. The inhibitory effects of propiconazole in this study were consistent with previous findings that abnormal plant growth was observed in rice brassinosteroid biosynthesis mutants (Hong, Ueguchi-Tanaka et al. 2005), lower lateral root density in rice mutants with the defective brassinosteroid signalling, *d61* (Nakamura, Fujioka et al. 2006), and propiconazole-treated maize and *Arabidopsis* (Hartwig, Corvalan et al. 2012, Best, Johal et al. 2017). These findings underly the

requirement of brassinosteroid in orchestrating plant growth and development. Brassinosteroid was previously reported that it interacts with auxin in the regulation of lateral root development in *Arabidopsis*. Brassinosteroid stimulates lateral root development at a low concentration via the promotion of auxin transport (Bao, Shen et al. 2004, Gupta, Singh et al. 2015). Recently, it was further demonstrated that brassinosteroid positively regulates and acts upstream of local auxin biosynthesis to promote lateral root elongation under low nitrogen in *Arabidopsis* (Jia, Giehl et al. 2021), indicating the existence of the interaction between the two plant hormones to regulate lateral root elongation. Therefore, the defective lateral root development was likely an effect of the propiconazole-induced deficiency of brassinosteroid on auxin-mediated mechanisms.

5.5 Effects of propiconazole on root diameter.

Propiconazole-treated roots were thicker than the untreated roots (figure 22 and 23), which is consistent with the previous studies that roots of the *Arabidopsis* mutants with the defects in brassinosteroid biosynthesis and wheat roots treated with brassinazole, a brassinosteroid biosynthesis inhibitor, also had thicker root diameter, while treatments of exogenous brassinosteroid resulted in thinner root diameter (Hou, Zhang et al. 2019, Vukašinović, Wang et al. 2021). This suggested that the observed effect of propiconazole on root diameter was likely due to the defects in brassinosteroid and the role of brassinosteroid in the regulation of root diameter is likely conserved among plant species, both monocots and dicots. Previous research in wheat showed that the number of cortex cell layer was not affected, but stele diameter, cell diameter, and the number of cells in each layer of epidermis, cortex, and endodermis were decreased and increased by exogenous brassinosteroid and brassinazole, respectively (Hou, Zhang et al. 2019). This could explain the effects of the propiconazole-induced deficiency of brassinosteroid that caused thicker root diameter. However, further investigation is necessary.

5.6 Effects of propiconazole on cell division and cell elongation.

Optimal root growth requires the balance between cell proliferation of root apical meristem and cell differentiation, including cell elongation, of newly-produced cells (Beemster and Baskin 1998, Takatsuka and Umeda 2014), which is tightly regulated by plant hormones including auxin and brassinosteroid (Takatsuka and Umeda 2014, Wei and Li 2016) Therefore, propiconazole could affect root meristem integrity, cell division, and cell elongation, leading to the inhibition of root growth in both primary and lateral roots. It has been demonstrated that propiconazole treatments reduced both meristematic and mature cell size, while ECS showed the opposite effects (Jantapo, Wimonchaijit et al. 2021), which is consistent with the well-studied role of brassinosteroid that positively regulates cell elongation (Oh, Honey et al. 2020, Vukašinović, Wang et al. 2021). Brassinosteroid regulates meristematic function both positively and negatively depending on its concentration (González-García, Vilarrasa-Blasi et al. 2011). In *Arabidopsis*, the mutants with the defects in brassinosteroid

signalling had lower cell division activity due to slower cell cycle progression (González-García, Vilarrasa-Blasi et al. 2011), and the mutants with the defects in either brassinosteroid signalling or biosynthesis had smaller meristem size with lower meristematic cell number and shorter meristematic cell length (González-García, Vilarrasa-Blasi et al. 2011, Li, Kang et al. 2020). Low concentration of brassinosteroid increased root meristem size, while it reduced root meristem size at the high concentration by causing premature differentiation of meristematic cells, leading to the inhibition of root growth. These previous findings indicated that optimal brassinoteroid response is required for optimal root growth (González-García, Vilarrasa-Blasi et al. 2011, Li, Kang et al. 2020, Jantapo, Wimonchaijit et al. 2021, Vukašinović, Wang et al. 2021), and could explain the inhibitory effects of high concentration of propiconazole and ECS observed in this study that severely inhibited root growth. However, it has been shown that the propiconazole-treated roots in rice at the moderate concentration at 4 µM in the previous study (Jantapo, Wimonchaijit et al. 2021) increased root meristem size with higher meristematic cell number. Together with the results of this study, the primary root of the rice seedlings treated with 5 µM propiconazole was not shorter (figure 23, 25 and 31) and roots treated with 5 µM propiconazole and ECS at low concentration, 1 and 10 nM, seemed to have longer primary root than those untreated (figure 33). This supported the possibility that the endogenous content of brassinosteroid in root meristem is supraoptimal (Jantapo, Wimonchaijit et al. 2021). Auxin is also required for maintaining the function of root meristem because the defects in local auxin biosynthesis or auxin content in root meristem can lead to the loss of meristem in both primary roots and emerged lateral root in *Arabidopsis* (Brumos, Robles et al. 2018, Ackerman-Lavert, Fridman et al. 2021). Therefore, the propiconazole-induced deficiency of brassinosteroid was likely to cause the defects in cell divion, cell elongation, and root meristem integrity, leading to the inhibition of the emergence of lateral root primordia and growth of both primary root and emerged lateral roots.

CHAPTER VI
CONCLUSION

This study demonstrated that propiconazole negatively affected the growth of rice seedlings by decreasing plant height, biomass, primary root length, and lateral root density. The shoot morphology of rice seedlings treated with propiconazole was similar to the brassinosteroid-deficient mutants, consistent with its ability to specifically inhibit the brassinosteroid biosynthesis. The results from this study further underlie the requirement of brassinosteroid for the optimal regulation of plant growth and development. Propiconazole caused the prominent decrease in emerged lateral root density. Unemerged lateral root primordia of the propiconazole-treated plants maturely developed with the structure similar to the mature root tip, and they were found in the root maturation zone far from the root tip. In addition, emerged lateral roots of the propiconazole-treated plants were also obviously shorter. These findings supported the possibility that propiconazole inhibits lateral root emergence and elongation, resulting in reduced emerged lateral root density. NAA treatment in the propiconazole-treated plants further indicated that the propiconazole-induced inhibition of lateral root development was possibly due to the defect in auxin-mediated mechanisms because NAA treatments were able to rescue emerged lateral root density in the roots of plants treated with propiconazole.

This study further provides greater information for understanding the potential adverse impact of propiconazole on the environment and assessing the risk of using propiconazole as a fungicide. In addition, the understanding of how root development is regulated in rice, additionally from the model plant *Arabidopsis*, will be beneficial for improving crop yields in the future.

APPENDIX

1. Statistical analysis of experiment 4.1

1.1 Descriptive statistics

Traits	Treatments	Number of replicates	Mean	Std. Deviation	Std. Error
Plant height (cm)	Control	15	18.5733	1.19431	.30837
	0.1 μM PPZ	15	17.3133	2.05457	.53049
	1 μM PPZ	15	13.4400	1.99993	.51638
	5 μM PPZ	15	11.8800	1.37176	.35419
	10 μM PPZ	15	10.0400	1.53055	.39519
	20 μM PPZ	15	8.0667	1.83563	.47396
Shoot fresh weight (g)	Control	15	.0820	.00721	.00186
	0.1 μM PPZ	15	.0801	.00908	.00234
	1 μM PPZ	15	.0656	.00899	.00232
	5 μM PPZ	15	.0604	.00927	.00239
	10 μM PPZ	15	.0472	.01023	.00264
	20 μM PPZ	15	.0375	.00746	.00193
Shoot dry weight (g)	Control	15	.0099	.00091	.00024
	0.1 μM PPZ	15	.0093	.00249	.00064
	1 μM PPZ	15	.0090	.00129	.00033
	5 μM PPZ	15	.0087	.00124	.00032
	10 μM PPZ	15	.0073	.00125	.00032
	20 μM PPZ	15	.0057	.00161	.00041
Root fresh weight (g)	Control	15	.0601	.01251	.00323
	0.1 μM PPZ	15	.0570	.00950	.00245
	1 μM PPZ	15	.0590	.01077	.00278
	5 μM PPZ	15	.0611	.00689	.00178
	10 μM PPZ	15	.0550	.00763	.00197
	20 μM PPZ	15	.0472	.00636	.00164

Traits	Treatments	Number of replicates	Mean	Std. Deviation	Std. Error
Primary root length (cm)	Control	15	7.6242	1.01253	.26143
	0.1 μM PPZ	15	6.8709	.52527	.13562
	1 μM PPZ	15	6.2354	.57562	.14862
	5 μM PPZ	15	7.5139	1.10318	.28484
	10 μM PPZ	15	6.5907	.78996	.20397
	20 μM PPZ	15	3.9185	.74180	.19153
Emerged lateral root density (lateral root number/ primary root length)	Control	15	22.7898	4.29817	1.10978
	0.1 μM PPZ	15	24.9189	3.60652	.93120
	1 μM PPZ	15	17.5430	3.54052	.91416
	5 μM PPZ	15	16.2956	2.86601	.74000
	10 μM PPZ	15	15.9860	3.80801	.98322
	20 μM PPZ	15	13.7741	5.30869	1.37070
Total root length (cm)	Control	15	160.3530	32.70393	8.44412
	0.1 μM PPZ	15	152.5136	29.24551	7.55116
	1 μM PPZ	15	98.0217	23.81857	6.14993
	5 μM PPZ	15	85.7163	21.46899	5.54327
	10 μM PPZ	15	54.9282	17.60974	4.54682
	20 μM PPZ	15	26.0130	9.19538	2.37424
Average root diameter (mm)	Control	15	.1990	.01509	.00390
	0.1 μM PPZ	15	.2016	.01676	.00433
	1 μM PPZ	15	.2465	.02444	.00631
	5 μM PPZ	15	.3020	.03598	.00929
	10 μM PPZ	15	.3747	.04969	.01283
	20 μM PPZ	15	.5058	.11674	.03014
Root surface area (mm^2)	Control	15	972.8782	173.53723	44.80712
	0.1 μM PPZ	15	932.6404	154.00409	39.76368
	1 μM PPZ	15	732.2427	138.02655	35.63830
	5 μM PPZ	15	778.5268	141.15431	36.44589
	10 μM PPZ	15	615.0495	144.31007	37.26070
	20 μM PPZ	15	383.2315	97.35360	25.13659

1.2 Test of Homogeneity of Variances

Traits	Levene Statistic	df1	df2	Sig.
Plant height	1.486	5	84	.203
Shoot fresh weight	.519	5	84	.761
Shoot dry weight	.765	5	84	.578
Root fresh weight	1.310	5	84	.268
Primary root length	1.510	5	84	.195
Emerged lateral root density	.923	5	84	.471
Total root length	2.533	5	84	.035
Average root diameter	6.944	5	84	.000
Root surface area	.613	5	84	.690

1.3 Anova: Single Factor

Traits		Sum of Squares	df	Mean Square	F	Sig.
Plant height	Between Groups	1258.902	5	251.780	87.621	.000
	Within Groups	241.376	84	2.874		
	Total	1500.278	89			
Shoot fresh weight	Between Groups	.023	5	.005	60.957	.000
	Within Groups	.006	84	.000		
	Total	.030	89			
Shoot dry weight	Between Groups	.000	5	.000	14.993	.000
	Within Groups	.000	84	.000		
	Total	.000	89			
Root fresh weight	Between Groups	.002	5	.000	4.595	.001
	Within Groups	.007	84	.000		
	Total	.009	89			
Primary root length	Between Groups	137.426	5	27.485	40.984	.000
	Within Groups	56.333	84	.671		
	Total	193.759	89			

Traits		Sum of Squares	df	Mean Square	F	Sig.
Emerged lateral root density	Between Groups	1410.282	5	282.056	17.830	.000
	Within Groups	1328.791	84	15.819		
	Total	2739.073	89			
Total root length	Between Groups	210444.293	5	42088.859	75.434	.000
	Within Groups	46868.462	84	557.958		
	Total	257312.755	89			
Average root diameter	Between Groups	1.058	5	.212	68.623	.000
	Within Groups	.259	84	.003		
	Total	1.317	89			
Root surface area	Between Groups	3535135.081	5	707027.016	34.458	.000
	Within Groups	1723559.838	84	20518.570		
	Total	5258694.919	89			

2. Statistical analysis of experiment 4.2

2.1 Descriptive statistics

Traits	Treatments	Number of replicates	Mean	Std. Deviation	Std. Error
Emerged Lateral Root Density (lateral root number/ primary root length)	Control	20	21.3884	2.77979	.62158
	5 μM PPZ	20	7.5579	3.43611	.76834
Total Lateral Root Density (Lateral root number/ primary root length)	Control	20	22.8292	2.01639	.45088
	5 μM PPZ	20	19.9950	1.93073	.43172
Lateral Root Primordia Density (Lateral root number/ primary root length)	Control	20	1.4408	1.56084	.34901
	5 μM PPZ	20	12.4372	3.07287	.68711
Primary Root Length (cm)	Control	20	6.9610	.71960	.16091
	5 μM PPZ	20	7.9426	1.25578	.28080

2.2 Student's T-Test

Traits		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Emerged lateral root density	Equal variances assumed	2.174	.149	13.995	38	.000	13.83055	.98828	11.82988	15.83123
	Equal variances not assumed			13.995	36.412	.000	13.83055	.98828	11.82701	15.83410
Total lateral root density	Equal variances assumed	.003	.959	4.540	38	.000	2.83419	.62424	1.57048	4.09790
	Equal variances not assumed			4.540	37.929	.000	2.83419	.62424	1.57040	4.09798
Lateral root primordia density	Equal variances assumed	18.962	.000	-14.269	38	.000	-10.99636	.77067	-12.55651	-9.43622
	Equal variances not assumed			-14.269	28.192	.000	-10.99636	.77067	-12.57453	-9.41820
Primary root length	Equal variances assumed	2.328	.135	-3.033	38	.004	-.98165	.32364	-1.63682	-.32648
	Equal variances not assumed			-3.033	30.263	.005	-.98165	.32364	-1.64236	-.32094

3. Statistical analysis of experiment 4.3

3.1 Descriptive statistics

Traits	Treatments	Number of replicates	Mean	Std. Deviation	Std. Error
Primary root length (cm)	Control	14	7.0804	.55001	.14700
	5 μM PPZ	15	8.3069	1.35806	.35065
	20 nM NAA	15	5.6314	.58447	.15091
	50 nM NAA	15	5.1988	.92043	.23765
	100 nM NAA	15	4.4447	.43919	.11340
	20 nM NAA+PPZ	15	7.0249	.60145	.15529
	50 nM NAA+PPZ	14	5.5792	.47316	.12646
	100 nM NAA+PPZ	15	4.4413	.57111	.14746
Emerged lateral root density (lateral root number/ primary root length)	Control	14	24.6143	1.60770	.42968
	5 μM PPZ	15	11.3112	3.64554	.94127
	20 nM NAA	15	30.7872	2.70569	.69861
	50 nM NAA	15	32.2942	5.06195	1.30699
	100 nM NAA	15	34.4653	2.74456	.70864
	20 nM NAA+PPZ	15	20.8291	7.28972	1.88220
	50 nM NAA+PPZ	14	29.8906	5.73919	1.53386
	100 nM NAA+PPZ	15	33.1811	3.89118	1.00470

3.2 Test of Homogeneity of Variances

Traits	Levene Statistic	df1	df2	Sig.
Primary root length	3.153	7	110	.004
Emerged lateral root density	3.786	7	110	.001

3.3 Anova: Single Factor

Traits		Sum of Squares	df	Mean Square	F	Sig.
Primary root length	Between groups	198.584	7	28.369	50.628	.000
	Within groups	61.638	110	.560		
	Total	260.221	117			
Emerged lateral root density	Between groups	6501.143	7	928.735	47.068	.000
	Within groups	2170.472	110	19.732		
	Total	8671.615	117			

4. Media composition

Stock no.	Chemical	g/l	Mw	Stock (M)	Working concentration (mM)
1	NH_4NO_3	91.400	80.04	1.1419	1.4274
2	$NaH_2PO_4.2H_2O$	40.300	156.01	0.2583	0.3229
3	K_2SO_4	71.400	174.26	0.4097	0.5122
4	$CaCl_2$	88.600	110.98	0.7983	0.9979
5	$MgSO_4.7H_2O$	324.000	246.52	1.3143	1.6429
6	$MnCl_2.4H_2O$	1.500	197.91	0.0076	0.0095
	$6(NH_4)\ Mo_7\ O_{24}\ 4H_2O$	0.074	1236.00	0.00005987	0.0000748
	H_3BO_3	0.934	61.83	0.0151	0.0189
	$ZnSO_4.7H_2O$	0.035	287.56	0.000122	0.000152
	$CuSO_4.5H_2O$	0.031	249.69	0.000124	0.000155
7	Fe(III)-EDTA	7.920	278.01	0.0285	0.0356

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VITA

NAME	Watcharapong Wimonchaijit
DATE OF BIRTH	16 May 1994
PLACE OF BIRTH	Samut Songkhram
INSTITUTIONS ATTENDED	Bachelor's degree from Chulalongkorn University, Bangkok
HOME ADDRESS	54/1, Thai Hat, Mueang Samut Songkhram District, Samut Songkhram 75000
PUBLICATION	Wimonchaijit, W. and Chaiwanon, J. 2021. Propiconazole inhibits root elongation and lateral root development in rice seedlings by interfering with auxin. Proceedings of the 47th International Congress on Science, Technology and Technology-based Innovation (STT47). 5-7 October 2021, pp. 394-399.