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To cite this article: Hajime Watanabe, Kae Honma, Yusuke Adachi & Akari Fukuda (2018) Effects of combinational treatment with ethephon and gibberellic acid on rice seedling growth and carbohydrate mobilization in seeds under flooded conditions, *Plant Production Science*, 21:4, 380-386, DOI: [10.1080/1343943X.2018.1520048](https://doi.org/10.1080/1343943X.2018.1520048)

To link to this article: <https://doi.org/10.1080/1343943X.2018.1520048>



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Published online: 22 Oct 2018.



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Effects of combinational treatment with ethephon and gibberellic acid on rice seedling growth and carbohydrate mobilization in seeds under flooded conditions

Hajime Watanabe^a, Kae Honma^b, Yusuke Adachi^b and Akari Fukuda^c

^aBioresource Sciences, Akita Prefectural University, Akita, Japan; ^bAgriculture, Niigata University, Niigata, Japan; ^cHokuriku Research Center, NARO Agricultural Research Center, NARO, Joetsu, Niigata, Japan

ABSTRACT

Early seedling growth, including seedling emergence and vigor, is crucial in direct seeded rice. In this study, we examined the effects of gibberellic acid (GA₃) and ethephon (ET) on the early growth of direct seeded rice, and on α-amylase activity and soluble sugar content in the seeds. Rice seeds were treated with water (control), ET, GA₃, or ET+ GA₃ and were allowed to grow for 21 days. The results showed that ET and GA₃ synergistically improved the lengths of mesocotyl, coleoptile, mesocotyl+ coleoptile, and first leaf. Moderate synergistic effects of ET and GA₃ were detected in the second and third leaves. ET+ GA₃ treatment induced higher α-amylase activities during early post-germination growth, which is consistent with the longer coleoptile, mesocotyl, mesocotyl+ coleoptile, and first leaf. In addition, ET+ GA₃ induced higher α-amylase activity in the seeds, and consequently, higher sucrose and glucose concentrations than other treatments. These results partially explain the regulatory mechanism underlying the synergistic interaction of ET and GA₃ on rice seedling growth in terms of starch and sugar metabolism in the seeds, and this knowledge is expected to facilitate the practical use of ET and GA₃ in direct seeded rice systems.

ARTICLE HISTORY

Received 12 April 2018
Revised 7 August 2018
Accepted 28 August 2018

KEYWORDS

α-amylase; coleoptile; direct seeding; ethephon; gibberellic acid; mesocotyl; *Oryza sativa* L

Introduction

Direct seeding is an attractive and sustainable method to reduce rice production costs because it reduces labor. Early seedling growth, including seedling emergence and vigor, is crucial in direct seeded rice systems. Seedling growth can be improved by treating the seeds with plant growth regulators (PGRs), such as gibberellin (GA), ethylene, and abscisic acid (ABA), which are known to affect the elongation of rice seedling organs (Furukawa et al., 1997; Raskin & Kende, 1983; Suge, 1974; Takahashi & Kaufman, 1983; Watanabe and Takahashi 1997; Watanabe et al., 2015, 2007). Suge (1974) reported that ethylene combined with GA₁, a naturally active GA in rice, stimulates the growth of the coleoptile and first leaf in rice. Furukawa et al. (1997) suggested that the synergistic effect of ethylene and GAs on leaf elongation could be attributed to an increased responsiveness and turnover of GA₁ by ethylene. Our previous study showed that simultaneous application of ethephon (ET) and gibberellic acid (GA₃) improved the early growth of rice at different growth temperatures and flooding depths under field and environmentally controlled conditions

(Watanabe et al., 2015, 2007), however, the physiological mechanism underlying the synergistic effect of ET and GA₃ on seedling growth remains unsolved, despite the above-mentioned report by Furukawa et al. (1997).

Vigorous seedling growth is crucial when rice seeds are sown in flooded paddy fields. For instance, the elongation of seedling organs, including mesocotyl, coleoptile, and first leaf, plays an important role in the emergence and establishment of direct seeded rice (Hoshikawa, 1993; Mori et al., 2012; Ogiwara & Terashima, 2001; Watanabe et al., 2015, 2007, 2006). Further, various physiological processes, including starch and sugar metabolism, are involved in the seedling growth of rice under flooded conditions (Ismail et al., 2009; Magneschi & Perata, 2009). For instance, numerous studies have demonstrated the importance of α-amylase in starch mobilization under oxygen-limiting conditions (Hwang et al., 1999; Ismail et al., 2009; Perata et al., 1997). However, it remains unclear whether physiological factors such as higher α-amylase activity and starch degradation are involved in the synergistic effects of ET and GA₃ on rice seedling growth.

In hypoxic conditions, coleoptile growth in rice is genotype-dependent (Ogiwara & Terashima, 2001; Setter & Ella, 1994). In young seedlings, the elongated coleoptile functions like a 'snorkel' by permitting contact with the air or aerated surface water, thus allowing continuous plant growth (Kordan, 1974). The physiological traits associated with coleoptile elongation of pre-germinated seeds under anoxia have been investigated (Setter & Ella, 1994). However, the mechanism of low-oxygen stress avoidance during post-germination growth and seedling emergence of ET and GA₃-treated seeds are not yet fully understood. Therefore, this study aimed to evaluate the physiological traits associated with synergy of ET and GA₃ on the growth of rice seedlings in waterlogged soils in light of the stimulation of α-amylase activity and the carbohydrate mobilization in seeds for early growth.

Materials and methods

Plant material and PGR treatment

The plant material used in this study was *Oryza sativa* L. ssp. japonica cv. Akidawara. The seeds were surface-sterilized in a solution of thiuram and benomyl for 24 h and soaked in water for 48 h. Seed sterilization and soaking were carried out at 15°C in darkness. Subsequently, the seeds were treated with ET (2-chloroethylphosphonic acid; Ishihara Sangyo Kaisha Ltd., Osaka, Japan), an ethylene-releasing compound (Arteca, 1996), and/or GA₃ (Sigma Chemical Co., MO, USA) as follows: the seeds were treated with one of the following solutions for 48 h: (1) water (control), (2) 250 mg L⁻¹ ET, (3) 20 mg L⁻¹ GA₃, or (4) 250 mg L⁻¹ ET + 20 mg L⁻¹ GA₃ (ET+ GA₃). The concentration of ET and GA₃ was determined by referring to those of Watanabe et al. (2007), and by preliminary experiments. After treatment, the seeds were briefly washed with water to remove any excess solution. PGR treatment and germination were carried out simultaneously at 30°C in the dark.

Culture conditions

The experiment was carried out under environmentally controlled conditions in a growth chamber as described previously by Watanabe et al. (2007), with minor modifications. Per treatment, 21 seeds were sown at a depth of 1 cm in compartmentalized seeding trays filled with fertilized granulated soil (Kureha Chemical Co. Ltd., Tokyo, Japan). After sowing, a common, 2-cm flooding depth treatment was applied and maintained throughout the growth period. Each experiment was replicated

four times. The seeds were allowed to grow at 20°C under a medium-day photoperiod (12 h light/dark). The growth chamber was illuminated by fluorescent lamps with a photosynthetic photon flux of 120 μmol m⁻² s⁻¹. The seedlings were allowed to grow for 21 days and then, plant height and the lengths of mesocotyl, coleoptile, mesocotyl+ coleoptile, and first to third leaves were measured.

Assessment of α-amylase activity and sugar content in seeds

To determine α-amylase activity and soluble sugar content in PGR-treated germinating seeds, we incubated the seeds at 20°C, as described above. The seeds were collected at 0, 2, 4, and 6 days after sowing (DAS), and the roots and hulls were removed with a razor blade. All samples were separated into the brown rice and plumule, immediately frozen in liquid N, and stored at -80°C until analysis. The brown rice samples were used in the present study. The α-Amylase activity and sugar content were assayed following the methods described by Fukuda et al., (2008), with slight modifications. The protein concentration in the extracts was measured using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). A Ceralpha kit (Megazyme Co., Ltd., Sydney, Australia) was used to assay α-amylase activity. The concentrations of soluble sugars (sucrose, glucose, and fructose) were determined using F-kits (J.K. International Co., Ltd., Tokyo, Japan). All above analyses were basically conducted following the respective instruction manuals.

Experimental design and statistical analysis

The experiment was set up as a randomized complete block design with four replications. Data were summarized as means and standard errors. Analysis of variance (ANOVA) followed by Tukey's test was used to identify significant differences between the treatments. $P < 0.05$ was considered significant. Statistical analyses were carried out using JMP 4.0. (SAS Institute, Cary, NC, U.S.A.).

Results

ET and GA₃ synergistically promote rice seedling growth

The application of ET and/or GA₃ affected all the parameters studied (Figure 1). ET+ GA₃ significantly increased the lengths of coleoptile, mesocotyl, mesocotyl+ coleoptile, and first leaf as compared to the control and treatment with ET or GA₃ alone. For instance, ET+ GA₃

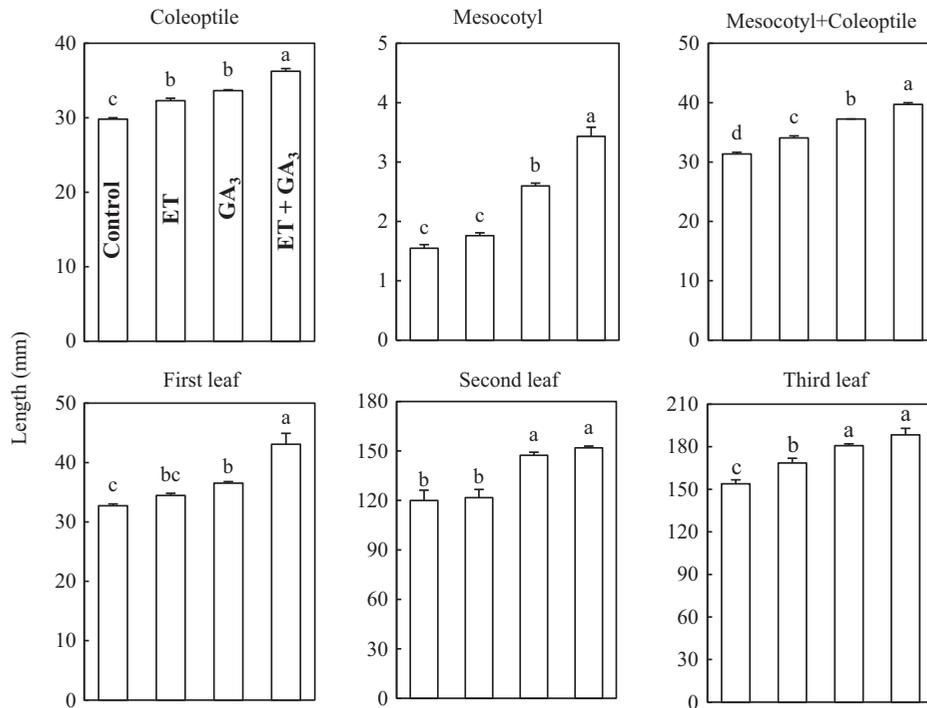


Figure 1. Effects of GA₃ and/or ET on the growth of rice seedlings. The seedlings were allowed to grow for 21 days. Bars represent the standard error of the mean for each treatment (*n* = 4). Different letters within each graph indicate significant differences between treatments as indicated by Tukey's test (*p* < 0.05).

increased mesocotyl+ coleoptile length by 26.7 %, 16.6 %, and 6.7 %, as compared to the control, ET, and GA₃ treatments, respectively. However, the effect of ET+ GA₃ on the second and third leaves was not significant when compared with that of GA₃ alone.

Next, we compared the growth curves of seedling organs under the different treatments (Figure 2). Mesocotyl+ coleoptile length was not different at 2 and

4 DAS among treatments, but it did significantly differ as of 6 DAS, with ET+ GA₃ having the strongest effect, between 6 and 12 DAS. The maximum length of mesocotyl+ coleoptile was approximately 40 mm under ET+ GA₃, whereas it was only 31 mm in control seedlings. Additionally, the elongation speed of mesocotyl+ coleoptile from 2 to 6 DAS was the highest under ET+ GA₃ among all treatments (Figure 2).

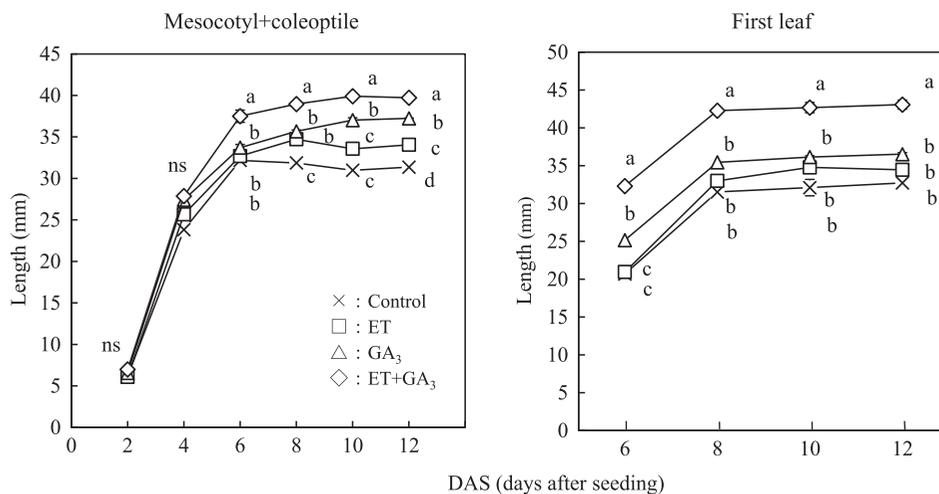


Figure 2. Effects of GA₃ and/or ET on mesocotyl+ coleoptile and first leaf length of rice seedlings. Bars represent the standard error of the mean for each treatment (*n* = 4). Different letters within each graph indicate significant differences between treatments as indicated by Tukey's test (*p* < 0.05). Comparison valid within same DAS.

The synergistic effect of ET and GA₃ was also observed in the growth kinetics of the first leaf (Figure 2). ET+ GA₃ significantly increased the length of the first leaf as compared to the control and ET or GA₃ treatments at 6 DAS. Elongation continued until 8 DAS under ET+ GA₃ treatment, and remained constant thereafter. Furthermore, the growth rate and final length were the highest under ET+ GA₃, similar to our observations for mesocotyl+ coleoptile.

ET and GA₃ synergistically increase α -amylase activity and sugar content in seeds

The α -Amylase activity was significantly higher under ET+ GA₃ than under the other treatments, even at seeding (0 DAS) (Figure 3). It slightly decreased at 2 DAS, and then increased progressively with time in all treatments, to reach a higher level at 6 DAS. The α -Amylase activity was the highest under ET+ GA₃ at all time points assessed. The α -Amylase activity under ET + GA₃ at 6 DAS was 1.2, 1.6, and 1.8 times higher than that under GA₃, ET, and control, respectively.

Time-course changes in soluble sugar contents in seeds are shown in Figure 4. Sucrose and glucose contents showed trends largely similar to that of α -amylase throughout the assessment period. The sucrose and glucose contents under ET+ GA₃ were generally the highest among treatments, with differences as compared to the control treatments being significant at 4 and 6 DAS. The sucrose content significantly increased under ET+ GA₃ as compared to the control; however, no significant difference was found between ET+ GA₃ and GA₃ or ET alone. The sucrose content under ET+ GA₃ at 6 DAS was 1.2, 1.4, and 1.6 times higher than that under GA₃, ET, and control, respectively. Glucose content under ET+ GA₃ significantly differed from that under

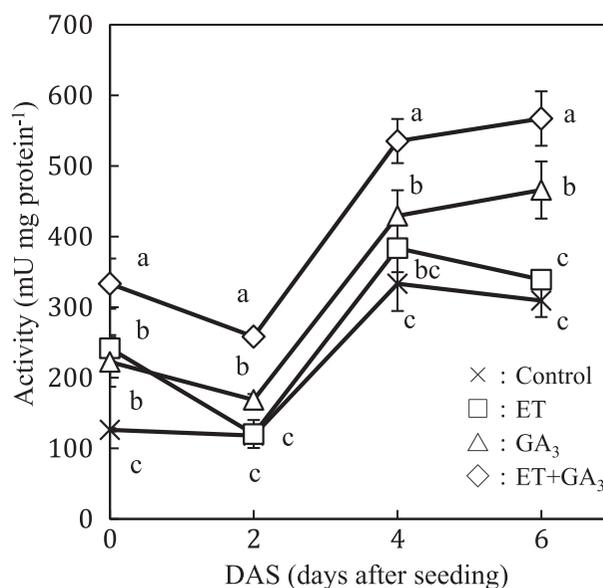


Figure 3. Effects of GA₃ and/or ET on α -amylase activity in seeds. Bars represent the standard error of the mean for each treatment ($n = 4$). Different letters within each graph indicate significant differences between treatments as indicated by Tukey's test ($p < 0.05$). Comparison valid within same DAS.

control and ET alone at 4 and 6 DAS. The glucose content under ET+ GA₃ at 6 DAS was 1.1, 1.1, and 1.2 times higher than that under GA₃, ET, and control, respectively. The amount of fructose in the seeds was generally lower than that of sucrose and glucose. No significant differences in seed fructose content were noted among the treatments throughout the experimental period.

Discussion

This study evaluated the physiological mechanisms underlying the synergistic effects of ET and GA₃ on

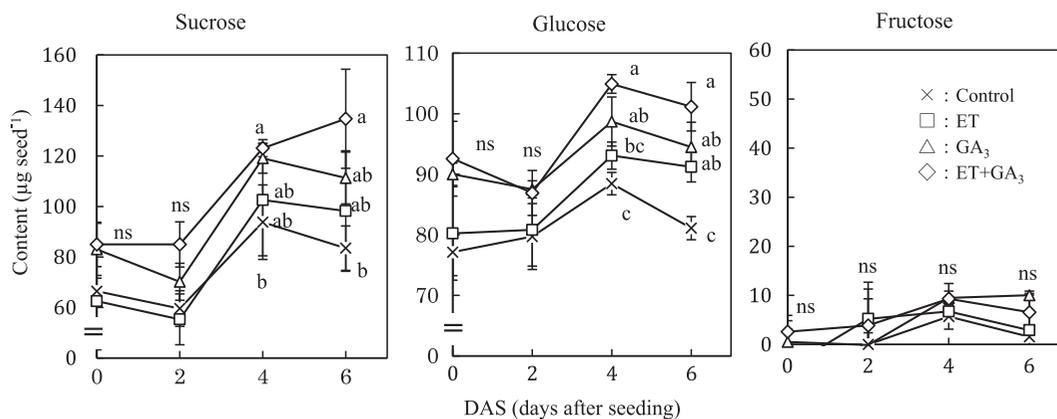


Figure 4. Effects of GA₃ and/or ET on soluble sugar contents in seeds. Bars represent the standard error of the mean for each treatment ($n = 4$). Different letters within each graph indicate significant differences between treatments as indicated by Tukey's test ($p < 0.05$). Comparison valid within same DAS.

rice seedling growth in terms of starch and sugar metabolism. We observed synergistic effects of ET and GA₃ on the lengths of coleoptile, mesocotyl, mesocotyl + coleoptile, and the first leaf, similar to previous findings using cv. Kokoromachi (Watanabe et al., 2007) and cv. Hitomebore (Watanabe et al., 2015); however, such synergistic effect was not observed in the second and third leaves in the present study. This suggests that the response of these two organs to ET and GA₃ might be cultivar-dependent (in the current study, cv. Akiawara was used) or that the magnitude of the response to ET and GA₃ might vary by organ. Indeed, the coleoptile, mesocotyl, and first leaf are primary and unique organs compared with the second and third leaves in terms of morphology and internal structure. Takahashi and Kaufman (1983) reported that the effects of PGRs are growth-stage-specific and organ-specific: for example, brassinolide and benzyladenine showed growth-promoting effects on early developing organs, such as the mesocotyl and coleoptile, while they had inhibitory effects on late-developing organs, including the second leaf blade. Another possibility is the difference in PGR treatment methods; in the current study, ET and GA₃ were applied at germination for convenience and ease of handling, while in the former studies, PGRs were added during seed soaking (Watanabe et al., 2015, 2007). Further study is needed for elucidating the observed differences in the synergy of ET and GA₃ on growth of rice seedling organs.

The growth curves of mesocotyl+ coleoptile and first leaf length under ET+ GA₃ were slightly steeper than those under other treatments (Figure 2). The elongation rate of mesocotyl+ coleoptile under ET+ GA₃ was the highest between 2 and 6 DAS, and that of first leaf was the highest between 6 and 8 DAS. These suggest that the fast elongation and longer length of mesocotyl + coleoptile and first leaf under ET+ GA₃ contribute to early seedling growth and vigor under flooding, because they are able to reach the soil surface, where oxygen is available. In accordance herewith, ET+ GA₃ positively affected the emergence and establishment of seedlings in field experiments (Watanabe et al., 2015).

There are several reports on the relationship between early seedling growth and physiological processes such as α -amylase activity and starch mobilization. Starch is a major energy source for post-germination growth, especially, in heterotrophic growth phases (Hwang et al., 1999; Ismail et al., 2009; Perata et al., 1997). For instance, rice genotypes tolerant to low-oxygen stress germinate and grow faster, and more seedlings survive. They maintain their ability to use starch reserves through higher α -amylase activity and anaerobic respiration (Ismail et al., 2009). We

previously reported that IR06F459, an AG (anaerobic germination) line with a long coleoptile, has high α -amylase activity and high sucrose and glucose contents in germinating seeds (Adachi et al., 2015). In the present study, higher α -amylase activity in the seeds was detected under ET+ GA₃ treatment (Figure 3). α -amylase activity was already significantly higher under ET + GA₃ than under the other treatments at seeding (0 DAS). This suggested that ET+ GA₃ treatment before seeding accelerates carbohydrate mobilization for subsequent growth. Additionally, ET+ GA₃ treatment induced higher α -amylase activities in early post-germination growth, which is consistent with the increased growth of coleoptile, mesocotyl, mesocotyl+ coleoptile, and first leaf. In addition, the higher α -amylase activity caused higher sucrose and glucose contents under ET + GA₃ than under other treatment (Figures 3 and 4). Although the present study focused on the effects of PGRs on rice seedling growth, our results are consistent with those of prior studies where the activity of α -amylases was higher in cultivars tolerant to low-oxygen conditions than in intolerant ones (Ismail et al., 2009, Adachi et al., 2015). The factors involved in the synergism of ET and GA₃ in rice seedling growth under waterlogged conditions remain unknown and require further study.

Synergistic plant hormone interactions have been found in several plant growth systems (Davies, 1995). A notable case of synergistic interaction is observed between ethylene and gibberellins in internode elongation in deep-water rice; ethylene promotes internode growth in response to flooding and increases the responsiveness of the internode tissue to gibberellins (Kende et al., 1998). Additionally, synergism of ethylene and gibberellins has been detected in rice coleoptile and first-leaf elongation (Suge, 1974; Watanabe et al., 2007, 2015), as well as in rice mesocotyl growth (Watanabe et al., 2015, 2007). Furukawa et al. (1997) attributed the synergistic action of ethylene and gibberellins on leaf elongation to the increased responsiveness and turnover of GA₁ by ethylene. A mechanism similar to that suggested by Furukawa et al. (1997) might underlie the synergistic action of ET and GA₃ on rice seedling growth; ET can increase the responsiveness of the seedling organ tissues to GA₃ or turnover of GA₁.

Gibberellin plays an essential role in starch mobilization by stimulating α -amylase activity (Jacobsen et al., 1995). Additionally, ethylene can augment starch hydrolysis by increasing the synthesis of and sensitivity to gibberellins (Ismail et al., 2009). The α -amylase activity in the seeds under ET+ GA₃ treatment maintained a high level during the growth period in the present

study (Figure 3). Considering the results of our study and the findings reported by Ismail et al. (2009), the synergistic action of ET and GA₃ is possibly due to enhanced amylase activity; it can be assumed that ET increased the GA₃-induced α-amylase activity.

Glucose and sucrose contents in the seeds were consistently higher under ET+ GA₃ than under other treatments (Figure 4); however, a clear synergistic interaction of ET and GA₃ was not detected. This might partially be due to simultaneous production and consumption of these substances; starch degradation in the seeds and utilization of soluble sugars for seedling growth occur concurrently. Takahashi et al., (2014) reported clear differences in soluble sugar concentrations in embryo between wild type rice and rad (*reduced adh activity*) mutant. If we measured soluble sugar concentrations of embryo and endosperm separately, as with the experiment of Takahashi et al. (2014), clear differences might be obtained among the PGRs treatments. In any case, the sugar availability for seedling growth under flooded soil conditions may be controlled by a highly complex mechanism that involves numerous physiological processes. Further research is warranted to clarify this possibility.

Multiple other enzymes are responsible for seedling growth under hypoxic conditions; for instance, ethylene is known to decrease peroxidase activity and consequently, peroxidase-induced lignification and protein assembly in the cell wall. Moreover, ethylene enhances sucrose transport from the scutellum to the growing coleoptile in germinating rice seeds, in which sucrose is cleaved into glucose and fructose (Ishizawa & Esashi, 1988); however, sugar availability is controlled by a highly complex mechanism that involves several gene regulatory pathways (Ismail et al., 2009; Ismail et al., 2012). Additionally, seedling growth under hypoxic conditions is involved in other essential physiological processes, including glycolysis and fermentation as well as starch breakdown (Bailey-Serres & Chang, 2005; Ismail et al., 2009, 2012). This study is a first step toward clarifying the physiological mechanisms underlying the synergistic effects of ET and GA₃ on growth of rice seedlings, therefore how these issues affect the synergy of ET and GA₃ deserves further experimental attention.

This study suggested that the growth regulation of target organs in rice seedlings will be possible by taking advantage of the synergism of ET and GA₃, which should have practical applications in rice direct seeding. Further studies will be needed to elucidate the particular roles of ethylene-gibberellin interactions and other regulatory processes in the growth of rice seedling under flooded soil conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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