Title: KODA, an α -ketol derivative of linolenic acid provides wide recovery ability of wheat against various abiotic stresses

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Abbreviations

KODA, 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid; CaCO₃, Calcium carbonate;

Ca(OH)₂, Calcium hydroxide

Abstract

In this study the application of α -ketol derivative of linolenic acid, KODA, was investigated in terms of root growth, plant development and crop production in response to CaCO_3 -imposed high pH (8.5) and drought, two major abiotic stresses for wheat production in Afghanistan. Two spring wheat cultivars, the Japanese cultivar Yumeshiho and a cultivar from Kihara Afghan wheat landraces (KAWLR) with long root (LR-504; KU11202Bb), were used in a CaCO_3 experiment. Another set of spring wheat cultivars, a KAWLR with short root (SR-823; KU7533), a KAWLR with long root (LR-744; KU7453), and a non-Japanese modern cultivar Lalmi-2, were used in the drought experiment. KODA significantly enhanced root elongation in Yumeshiho under CaCO_3 -imposed alkalinity conditions. For the drought experiment, KODA significantly increased seed germination in all cultivars. KODA-treated plants had increased root and shoot growth in normal culture conditions and greater recovery from drought, with the greatest root length in Lalmi-2 (43-51%). Interestingly, KODA-treated plants tends to increase production, in terms of an increase in productive tillers, number of grains per spike, and grain weight per plant. The recovery rates for plants that received KODA, in terms of grain weight per plant, were 2.5–3% in Lalmi-2, 13–15% in SR-823, and 11–13% in LR-744. In preliminary investigation, KODA-treated root showed moderate increasement and stability in wheat root expansisn genes under normal culture condition. These KODA-mediated physiological traits could be useful to efficiently uptake water and nutrients, and promote stable production in response to drought and high carbonate alkaline

soils worldwide.

Key words: KODA, Drought, Heat, CaCO₃ alkalinity, Wheat

1. Introduction

The major abiotic stresses for wheat cultivation in the rain-fed region in Afghanistan are drought, heat, CaCO₃-imposed alkalinity, and high pH. With respect to drought, limited root growth affects efficient uptake of water and nutrients, thereby inhibiting overall growth and productivity (Wasson et al., 2012). In the rain-fed region, the hypothesis is that a deeper and longer root system increases the water uptake from depth of the soil. CaCO₃-imposed alkalinity and with high pH, are another issue in Afghanistan that have not been given sufficient attention by the scientific community. Many vascular plant species are unable to successfully colonize in CaCO₃ sites, due to morphological and physiological alterations (Haque et al., 2015; Mabey, 1996; Conti et al., 1999; Lee, 1999; Ström et al., 2005) resulting in lower yield and quality. Wheat yield is reduced widely when grown under calcareous soils (Cakmak et al., 1999; Cakmak et al., 2001).

Due to the civil war, in the last three decades all agricultural research activities in Afghanistan were terminated and, until now, reports on these issues in wheat have been minimal. Therefore, we are at a moment in the history when a great transformation is needed to respond to immense threats to Afghanistan agriculture. Since disturbed physicochemical soil properties result

in an imbalance in plant resources and nutrition absorption, without proper balance in root growth and plant development, it would be difficult to improve productivity under abiotic stress conditions. Agricultural scientists must conduct research to address these issues.

We are certain that plants would show more improved growth and productivity if one or more of the root and seedling traits could be improved at early stages of stress imposition. We observed the effect of 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid (KODA), the α -ketol derivative of linolenic acid (Feussner and Wasternack, 2002; Mizuno et al., 2003), on the growth of wheat plants when grown under the above-mentioned conditions. KODA was first found to be a stress-inducing substance derived from duckweeds (Yokoyama et al., 2000). KODA reacts with catecholamine and acts as a flower-inducing factor in *L. paucicostata* “151” (Yamaguchi et al., 2001). KODA was discovered to improve flower formation in ornamental species (Yokoyama, 2010; Ono et al., 2013) and in fruit trees (Kittikorn et al., 2011; Nakajima et al., 2011). KODA seems to suppress the process of entering into dormancy (Yokoyama, 2010). Furthermore, Sakamoto et al. (2010) reported that KODA promote the breaking of dormancy of apical buds in pear (*Pyrus spp. L.*). KODA enhances adventitious root re-differentiation from *Swertia japonica* callus (Kawakami et al., 2015) and the rate of rooting from stem cuttings of Japanese Yamazakura (*Prunus serrulata*) (Yamamoto, 2010) and Kunugi (*Quercus acutissima* Carruth) plants (Yamamoto and Hakamada, 2008). Thus, KODA tends to be involved in the recovery from adverse effects due to stresses, but not in stress imposition.

In the present paper, various dosages of KODA were applied to seeds of some spring type Kihara Afghan wheat landraces (KAWLR), and one Japanese and one non-Japanese spring type wheat cultivar. The KODA-treated seeds were grown in cups containing CaCO_3 to induce high pH (Haque et al., 2015), and in the drum can method to induce drought. Data were collected on root and seedling physiological traits for the cup method experiment. For the drum can method experiment, KODA-treated plants were examined for seed germination, seedling survivability, root growth, shoot growth, yield, and yield component traits, compared with plants without KODA treatment.

2. Materials and Methods

2.1. Plant materials

For the CaCO_3 -imposed alkalinity and high pH (8.5) experiment, we used the Japanese spring wheat cultivar Yumeshiho and the Afghan landrace long root-504 (LR-504; KU11202Bb) spring wheat cultivar. For the drought experiment in the drum, we used one leading modern wheat cultivar (Lalmi-2) grown in Afghanistan and released by CIMMYT, and two spring type landraces; one cultivar from the long root-744 (LR-744; KU7453), and one short root-823 (SR-823; KU7533) landrace.

2.2. CaCO_3 alkalinity coupled with high pH (8.5) experiment in two-chambered cup

Saturated CaCO_3 (Wako Pure Chemical Industries, Ltd.) solution preparation and pH measurement were done according to our previous report (Haque et al., 2015). Seeds were imbibed with 5 μM and 10 μM of KODA, which was provided by Shiseido Co., Kakegawa, Japan, for overnight. Thirteen healthy seeds were sown on culture ball soils in the upper cup of the double-chambered transparent plastic cup system (upper cup: height 11 cm $\times$ upper diameter 8.5 cm, and bottom diameter 5.2 cm; bottom cup: height 12 cm $\times$ upper diameter 8.0 cm, and bottom diameter 5.2 cm) (Haque et al., 2015). The cups were placed in a rectangular plastic box containing 16-pockets, covered with dark sheets and transferred to a growth chamber maintained at a temperature of approximately 22°C day/20°C night, at an humidity approximately 44% day/28% night, with the natural light (intensity approximately 700-1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 12 h day length) in 2014 and 2015. Seedlings were thinned to 10 per cup and boxes were rotated every day to minimize the position effects on plant growth. High CaCO_3 and high pH treatments, and application of Hyponex fertilizers, were performed according to Haque et al. (2015). Plants were harvested 35 days after initiating the CaCO_3 treatment. After removing the lower cup, the roots were carefully separated. The plants were then carefully removed from the upper cup, followed by washing in water. Root length was measured only for root axes, not including branch roots. Shoots and roots were oven dried for 2 days at 80°C, and the dry weights were recorded. Experiments were repeated twice, with four replicates. Data were summarized by calculating means and standard errors, and analyzed by analysis of variance (ANOVA).

2.3. Drought stress experiment in drum can

Large steel drum cans (height 85.5 cm × diameter 60 cm) were used to impose drought condition. The upper 5.5 cm of the drum was air space. The bottom 5 cm of the drum was filled with perlite (IRISOHYAMA INC., Japan). The drums were filled with non-nutritious lawn soils (Kobayashi Sangyo Co. Ltd., Osaka, Japan). The top 20 cm soil of the drum was mixed with fertilizer. Each drum had eight 1-cm diameter holes drilled 3-cm above the bottom, so that the water could enter the drum as the control. Each drum was partitioned into three equal chambers, with black plastic boards. Seeds were imbibed with 0, 1 and 5 μM of KODA for 2 overnights.

Six healthy seeds for each concentration were sown at a 3-cm soil depth and divided into three slots (two seeds/slot). Therefore, each drum had 0 μM of KODA in its first chamber, 1 μM in the second chamber, and 5 μM in the third chamber. Seeds were sown on December 31, 2014 and allowed to germinate until mid-January 2015, without irrigation. Germination was reduced, particularly for seeds not treated with KODA. Then, the experiment was divided into a normal set, where soil inside the drums received water from the top and 10 cm of water at the bottom, using double layers of plastic and wood.

For the drought set, irrigation was applied only from the top and moisture was controlled within 20 cm of the soil surface. For the moisture measurement, we used a Daiki pF meter 04 (Daiki Rika Kogyo Co., Ltd., Saitama, Japan) at 10, 20, and 40 cm soil depths. The experiment was conducted inside a controlled plastic house at the Kihara Institute for Biological Research

(KIBR), Yokohama City University (YCU), with temperatures of $20 \pm 2^{\circ}\text{C}/10 \pm 2^{\circ}\text{C}$ (day/night) and $25\text{--}30 \pm 2^{\circ}\text{C}/15\text{--}20 \pm 2^{\circ}\text{C}$ (day/night) for December–April and May–June, respectively.

Plants were harvested on June, 2015 at physiological maturity. After recording the number of productive tillers (PT), the shoots of each slot in an individual chamber were first separated and then bound with rope. After recording the KODA concentration on tape, the drum was then carefully placed on plastic on the ground. The soil was removed carefully from the individual chambers. The plants were then removed carefully and photographed. Root length was measured only for root axes, i.e., not including branch roots. Shoots and roots were oven dried for 2 days at 80°C , and dry weight was recorded. Experiments were repeated in four biological repetitions, with three replicates.

After measuring the spike lengths (SL), spikes from each plant were cut and placed in onion bags. The bags were then suspended for a few weeks to dry with a large fan. After thrashing, the numbers of grains per spike (NGPS), grain weight per spike (GWPS), grain weight per plant (GW/plant), and thousand grain weight (TGW) were recorded. Data were summarized by calculating means and standard errors, and analyzed by ANOVA.

2.4. Calculation of germination percentage and recovery rate of growth parameters

The germination percentage of wheat were calculated using the relation shown below.

$$\text{Germination (\%)} = \frac{\text{Total seeds germinated}}{\text{Total seeds planted}} \times 100$$

For recovery rate, the first relatively increased or decreased rates were calculated by subtracting the values of the control from drought of non-treated KODA plants. Then, the recovery rate was calculated from the values of KODA-treated plants grown under drought, using the following formula. n= Plant from normal condition without KODA-treatment d= Plant from drought condition without KODA-treatment dKODA = Plant from drought condition with KODA-treatment

$$\text{Recovery rate (\%)} = \left(\frac{n-d}{n} \times 100 \right) - \left(\frac{n-d_{\text{KODA}}}{n} \times 100 \right)$$

2.5. RNA extraction and Semi-quantitative reverse transcriptase PCR (RT-PCR)

Seeds of Japanese wheat cultivar Yumeshiho were treated with 0, 1 and 5 μM of KODA followed by grown on two-chambered cup methods as described in the materials and methods section 2.2. When the first leaf was immersed completely, whole root (three seminal roots) was sampled by cutting out from shoot part. This sampling was called as initial. From this time point, sampling were done at week 1 when two leaves were completely opened and third leaf was partly opened (five roots; three old with two newly emerged seminal roots), at week 2 when three leaves were completely opened and fourth leaf was partly opened (five roots; three old and two elongating newly emerged seminal roots) and at week 3 when four leaves were completely opened and fifth leaf was partly opened (five well grown seminal roots).

The frozen root tissues (100–400 mg) were ground into fine powder under liquid nitrogen with a mortar and pestle. Total RNA was extracted by using a FavorPrep™ Plant Total RNA Mini Kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan). All samples were treated with RNase-free DNase I (Qiagen). 1 µg of total RNA isolated from each sample was reverse-transcribed to cDNA in a 20-µL reaction volume by using a SuperScript® II RT reagent kit (Invitrogen). Semi-quantitative RT-PCR was performed on the cDNA product corresponding to 20 ng of total RNA using an Ex Taq PCR kit (Takara). The PCR conditions were as follows: 30 cycles of 98°C for 10 s, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 m and 20 s, with a final extension at 72 °C for 7 m. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel stained with ethidium bromide. After taking image, the fluorescence signal was calculated using the Typhoon 9400 Variable Mode Imager coupled Image QuanTL program (GE Healthcare, CA, USA). An *actin* gene was used as an internal control (forward: 5'-ctatgttcccggtattgct-3' and reverse: 5'-aaggaggagcaagaatcgac-3') (Haque et al., 2014). The sequences of specific primers for *TaEXPA4*, *TaEXPA5*, *TaEXPA8*, *TaEXPA9*, *TaEXPB8* and *TaEXPB10* were used from previous report (Lin et al., 2005). All semi-quantitative RT-PCR experiments were performed in biological duplicate and technical triplicate.

3. Results

3.1. Recover root and seedling growth of plants treated with KODA, as affected by CaCO_3 -imposed alkalinity and high pH

When grown under CaCO_3 alkalinity, the root length and dry weight were slightly increased in the modern Japanese wheat cultivar Yumeshiho (Figs. 1 and **Supp. Fig. 1A**). Interestingly, KODA significantly enhanced this root elongation in Yumeshiho, under normal water (pH-7.5) and CaCO_3 solution. For the cultivar LR-504, KODA-treated plants were not significantly different from the KODA non-treated plants, but root dry weight was slightly reduced for the CaCO_3 treatment (Figs. 1 and **Supp. Fig. 1A**). Above ground **parameters were not significantly affected by KODA treatment (Figs. 1 and Supp. Fig. 1B).**

3.2. Developing drought conditions in the drum can

The initial goal of this research was to establish a methodology to simulate drought in a large drum can (**Fig. 2A**) that is between proxy laboratory and real field conditions. In this study, the scientific methodology was established and confirmed with one modern Lalmi-2 and two KAWLR wheat cultivars. Our pF meter data clearly showed (**Figs. 2B, C**) that, compared with the moisture drum condition, we successfully controlled water movement within the top 20 cm soil layer in the drought drum, as it naturally occurs in farmers' fields. Again, the pF meter data at the 40 cm depth confirmed the dry zone after 20 cm.

3.3. Recovery role of KODA on seed germination

KODA increased seed germination by 5–10% for Lalmi-2 and by 0–5% for SR-823 and LR-744, under normal conditions (Fig. 3). The KODA treatment showed recovery rates from dry soil of 13–23% for Lalmi-2, 5–24% for SR-823 and 13–24% for LR-744.

3.4. Role of KODA in root and shoot growth balance under drought

We investigated if KODA has a role in recovering the balance of the root and shoot growth, which are usually affected by drought. For normal conditions, root and shoot length were slightly increased, and root and shoot drymass were highly increased when KODA was applied (Figs. 4 and 5). As calculated using the formula stated in the materials and methods section, under drought conditions, KODA was able to recover 43–51% of the root length, 1–13% of the shoot length, 20–62% of the root biomass, and 6–25% of the shoot biomass for Lalmi-2. For SR-823, the recovery rates were 21–27% for root length, 0–10% for shoot length, 0–16% for root biomass, and 29–54% for shoot biomass. For LR-744, the rates were 13–27% for root length, 7–10% for shoot length, 11–45% for root biomass, and 31–64% for shoot biomass.

3.5. Contribution of KODA for yield improvement under drought

Since the ultimate goal of this study was to investigate the application of KODA, we examined yield performance of KODA-treated plants. For the normal drum condition, the yield parameters

were not significantly affected by KODA treatment (Table 1 *upper panel*). For the drought drum condition, SL, GWPS and 100 GW were not significantly affected by KODA treatment (Table 1 *lower panel*). PT, NGPS and GW/plant were significantly affected by KODA treatment (Table 1 *lower panel*). We have calculated the recovery rate by KODA-treated plants under drought condition comparing with KODA non-treated plants using a formula (as described in the materials and methods section). The rates for KODA-treated plants recovering from drought were 23–25%, 20–21%, and 0–17% for Lalmi-2, SR-823, and LR-744, respectively, for PT (Supp. Table 1). For NGPS, the recovery rates were 2–3%, 18–20%, and 19–20% for Lalmi-2, SR-823 and LR-744, respectively. For GW/plant, the recovery rates were 2.5–3%, 13–15%, and 11–13% for Lalmi-2, SR-823, and LR-744, respectively.

3.6. Expansin gene expression in roots during seedling development

The time-course expression of wheat expansin genes in whole roots during seedling development was examined by semi-quantitative RT-PCR from KODA-treated and KODA non-treated seeds. Out of eight wheat expansin genes, two were not amplified in the roots of Yumeshiho and none of them were expressed in leaf except *TaEXPA9* (Fig. 6 and Supp. Fig. 5). Primer efficiency were conducted with 25, 30, 35 and 40 cycles of PCR (Supp. Fig. 4). As observed, except *TaEXPA9*, all the expansins were well expressed in root when compared to actin. The *TaEXPA4* mRNA level was increases gradually during seedling development until 3 weeks for 5 μ M KODA compared with

0 μ M and 1 μ M KODA where the expression increases until 1 week and then decreases as seedling development progresses (Fig. 6). In addition, *TaEXPB10* showed slight increase in expression for 5 μ M KODA until 3 weeks, when the experiment was concluded (Fig. 6). The expression of *TaEXPA5*, *TaEXPA5*, *TaEXPA9* and *TaEXPB8* were almost remained unchanged (Supp. Fig. 5).

4. Discussion

We report KODA mediated root growth, plant development, and production against drought and CaCO₃ alkalinity, plus high pH (8.5). These sources of abiotic stress are the two major agricultural constraints in wheat cultivation in Afghanistan.

Our previous study included a semi-hydroponic wheat culture system, involving CaCO₃ and 8-d-old plants of a modern wheat Lalmi-2 cultivar. There was a moderate reduction in root and shoot growth under CaCO₃ alkalinity (Haque et al., 2015). Interestingly, most of the KAWLR showed almost no reduction in (and sometimes increased) root and shoot growth under CaCO₃ alkalinity (Haque et al., 2015). KODA had a positive effect on root elongation, particularly in the modern Japanese wheat cultivar Yumeshiho, in response to normal water (pH-7.5) and CaCO₃ solution (Figs. 1 and Supp. Fig. 1), suggesting that KODA alters root architecture and produces longer roots. This positive impact on root architecture has extensive applications in agriculture. Longer roots have a greater chance of encountering and exploiting resources, particularly during drought, as water reserves are typically located deeper in the soil profile (Manske and Vlek, 2002;

Wasson et al., 2012; Chapagain et al., 2014). High CaCO_3 is commonly found in dry soil (FAO, 1972) and, thus, the altered root architecture derived by KODA would be directly applicable in dry and high CaCO_3 soils, such as the type of soil found in Afghanistan. However, at present, we do not know the precise mechanism of the longer root architecture induced by KODA. Since CaCO_3 application could not induce a pH greater than 8.5 (Haque et al., 2015), we also examined the performance of KODA under Ca(OH)_2 -imposed very high pH (11.0), and found that root and shoot growth remained unchanged in the KODA-treated plants when compared to plants not treated with KODA (Supp. Fig. 2). One of the reasons for this response might be because the pH level (11.0) we induced with Ca(OH)_2 application was too extreme. We are currently checking the recovery effects from KODA application in response to a series of pHs imposed by Ca(OH)_2 treatment.

In our investigation, the most remarkable yield phenotypes of the KODA-treated plants under drought conditions were the number of productive tillers, number of grains per spike, and final yield (grain weight/plant; Table 1). In the field, drought first affects roots and limits uptake of water and nutrients, thereby inhibiting wheat development and final production (Wasson et al., 2012). Therefore, yield could be improved if KODA-treated plants have enhanced root and seedling growth at early growth stages. As expected, KODA-treated seed showed a greater recovery in seed germination (Fig. 3) and seedling growth (Fig. 2) compared to the KODA non-treated plants.

In our drum culture system, we sowed seeds after treating with KODA at 3 cm soil depth

and no irrigation was applied until after 1 month (Fig. 2C). Therefore, the greater seed germination recovery of KODA-treated seeds (Fig. 3) indicated that KODA altered seed physiology, which enhanced seed germination and seedling vigor during the first month. This alteration made the seedlings from KODA-treated seeds vigorous at the early growth stages, which contributed to enhanced root and shoot development (Figs. 4 and 5) in the water-limiting zone, from 30 cm to the bottom of the drum (Fig. 2A). This confirming that KODA have an impact on wheat stability in response to drought, however, the mode of KODA action is still an additional step to confirm. In a preliminary investigation, 5 μ M KODA-treated plants showed increased and stable expression in some expansin genes particularly, *TaEXPA4* during early seedling development (Fig. 6 and Supp. Fig. 5). In our experiment, *TaEXPA4* expression were gradually decreased in the 0 and 1 μ M KODA-treated roots when the seedling development increased (Fig. 6). In this experiment, we used whole roots from initial, 1, 2 and 3 weeks of seedling development. After grinding into powder, we used 100 mg root tissues for total RNA extraction. Therefore, one explanation is that *TaEXPA4* might be tissue specific (probably root tip tissue) and when the number of whole root tissues increase, *TaEXPA4* containing tissues proportionally decrease with the seedling development progressed. On the other hand, *TaEXPB10* is most probably widely expressed gene in whole root tissues. The current study was done only under normal condition. Authors are now conducting experiment on stress-grown roots as well as tissue-specific expression and localization of not only observed wheat expansins but also other target genes using root-anatomy coupled

micro-dissection and *in situ* hybridization technology. Expansins are multi-gene families and are reported to be participate in a diverse range of developmental processes mainly related to the action of cell wall loosening such as seed germination, growth rate, meristem dynamics, root development, root hair emergence, stem elongation, fruit softening, as well as in adaptive responses to submergence, gibberellin and wounding (Cosgrove, 2000; Lin et al., 2005; Hu et al., 2013; Hu et al., 2014). It has been demonstrate that epigenetic modifications contribute to the expression divergence of three *TaEXPAI* homologs during wheat development (Hu et al., 2013). Thus, there is greater possibility of KODA-triggered epigenetic effect on expansin expression for wheat stability against stress recovery. Moreover, the possibility of plant hormone such as auxin, cytokinin, gibberellin-mediated root development and greater plant growth in KODA-treated wheat plants (Figs. 4 and 5) cannot be ignored. Furthermore, the alteration of endogenous reactive oxygen species (ROS) and, thereby, gibberellin levels (Kim et al., 2007), for greater branching in KODA-treated plants (Table 1) must be clarified. KODA contributes to root and flower improvement in ornamental and tree plants (Yokoyama, 2010). A more general role in the defense system, and an impact on physiological phenotypes, is expected, considering the relationship between minimum inputs and greater physiological outputs, including the agronomic potential of plants. Although in wheat fields, drought and heat occur simultaneously, in our experiment KODA-treated plants remained unchanged in yield and yield-contributing traits (Supp. Fig. 3 and Supp. Table 2). The differences observed in these traits are mainly due to increases in related traits,

before the initiation of drought and heat. The reason for this phenomenon is unclear; further study is required of the interaction of KODA with heat, such as a heat experiment under conditions independent from drought. To clarify these issues, we also conducted experiments in real fields in Afghanistan and Japan by comparing KODA-treated plants with non- KODA-treated plants.

Given these results, how does KODA mediate regulatory circuits in wheat in response to CaCO_3 -imposed alkalinity and drought? We have proposed a possible model to explain the mode of KODA action for greater root growth and plant development, which might lead to improved yield and yield contributing traits (Fig. 7). Applying KODA during seed imbibition is hypothesized to cause direct, or certain endogenous, alterations, leading to epigenetic effects related to various physiological pathways, including ROS or the plant-hormone biosynthesis pathway. Thus, epigenetics might cause greater seed germination, and root and shoot growth, until flowering. These responses favor KODA-treated plants when grown under conditions of CaCO_3 -imposed alkalinity (*above panel*) and drought (*below panel*) stress, and improved final production.

5. Conclusions

Wheat genotypes treated with KODA showed specific positive responses to normal culture, and recovery from drought and CaCO_3 application. Morphological and physiological traits, such as longer root architecture, was key benefits for KODA-treated Japanese Yumeshiho under CaCO_3 treatment, while early recovery from low germination – or less seedling vigor caused by drought,

which improved root and shoot growth – were the responses of KODA-treated Lalmi-2, SR-823, and LR-744 plants in dry soil conditions. We **have to** clarify the precise molecular basis of KODA-triggered responses in plants. Moreover, the performance of plants treated with KODA, in response to other stresses, such as salinity, **need to be** investigated.

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Tables

Table 1

Yield and yield contributing traits under drought condition. Percentages indicate the rate of reduction due to KODA treatment. Single and double asterisks indicate significant differences at the 5% and 1% level by analysis of variance, respectively. ns, not significant.

	Treat						
Cultivars	ments	PT	SL	NGPS	GWPS	GW/plant	1000 GW
Normal							
Lalmi-2	0μM	10.7±0.8	12.7±0.2	63.0±1.5	1.33±0.1	14.2±0.1	22.0±0.3
	1μM	12.3±0.2 (15%)	13.0±0.2 (1%)	69.0±0.6 (10%)	1.45±0.1 (9%)	16.1±0.9 (13%)	23.9±0.6 (8%)
	5μM	11.6±0.7 (9%)	13.0±0.1 (1%)	68.3±0.1 (9%)	1.46±0.2 (10%)	15.67±1.2 (10%)	24.33±1.3 (10%)
SR-823	0μM	14.7±1.3	14.5±0.1	22.2±1.8	0.65±0.03	9.55±0.3	27.2±1.2
	1μM	16.1±1.4 (10%)	14.7±0.1 (1%)	24.3±2.6 (9%)	0.71±0.02 (9%)	10.5±0.5 (10%)	28.67±0.17 (5%)
	5μM	16.8±0.7 (14%)	15.3±0.2 (5%)	24.4±2.6 (10%)	0.72±0.03 (11%)	10.6±0.9 (11%)	28.67±0.18 (5%)
LR-744	0μM	16.3±0.3	14.8±0.1	15.33±0.7	0.67±0.03	10.85±0.5	33.3±0.35
	1μM	18.3±0.6 (12%)	15.3±0.2 (3%)	16.9±0.6 (10%)	0.73±0.03 (9%)	11.87±0.5 (9%)	35.53±1.3 (7%)
	5μM	18.7±1.5 (15%)	15.6±0.2 (5%)	17.0±0.7 (11%)	0.74±0.02 (10%)	11.93±1.2 (10%)	35.73±0.73 (7%)
Treatment (T)		ns	ns	ns	ns	ns	ns
Cultivar (CV)		*	ns	**	**	ns	**
T×CV		ns	ns	ns	ns	ns	ns
Drought							
Lalmi-2	0μM	9.0±2.3	8.9±0.2	6.0±0.6	0.22±0.02	1.94±0.1	20.00±1.15
	1μM	11.7±1.2 (30%)	10.1±0.1 (13%)	7.8±0.6 (30%)	0.25±0.03 (15%)	2.39±0.3 (23%)	23.00±2.0 (15%)
	5μM	11.5±1.8 (28%)	9.9±0.1 (11%)	7.5±0.1 (25%)	0.24±0.03 (14%)	2.35±0.2 (21%)	23.26±1.27 (16%)
SR-823	0μM	11.0±0.6	11.6±0.2	16.4±1.5	0.63±0.03	6.63±0.4	25.66±2.9
	1μM	14.0±0.6 (27%)	12.6±0.1 (9%)	20.5±2.5 (25%)	0.72±0.04 (13%)	7.80±0.4 (18%)	28.2±1.5 (10%)
	5μM	13.9±1.0 (26%)	13.3±0.2 (15%)	20.8±1.2 (27%)	0.74±0.05 (17%)	8.0±0.7 (21%)	28.93±1.8 (13%)
LR-744	0μM	10.7±1.3	12.0±0.2	10.7±0.9	0.57±0.03	7.0±0.6	27.13±1.6
	1μM	13.5±0.6 (26%)	12.7±0.1 (9%)	13.6±0.9 (27%)	0.65±0.03 (14%)	8.3±0.7 (19%)	31.00±0.6 (14%)
	5μM	13.5±0.6 (26%)	13.6±0.1 (15%)	13.8±0.6 (29%)	0.66±0.02 (16%)	8.5±0.3 (21%)	31.33±1.8 (15%)
Treatment (T)		*	ns	*	ns	*	ns
Cultivar (CV)		ns	ns	**	**	**	*
T×CV		ns	ns	ns	ns	ns	ns

PT, Productive tillers per plant; SL, Spike length per plant; NGPS, Number of grain per spike; GWPS, Grain weight per spike; GW,

Grain weight

Legends to figures

Figure 1. Morphology of the modern wheat cultivars Yumeshiho and LR-504 grown for 35 days under CaCO_3 -induced alkalinity and high pH (8.5) conditions. (A) KODA-treated and KODA non-treated plants in the representative two-chambered cups. (B) Roots after harvesting from two-chambered cups. The photograph shows a representative image of four replicates.

Figure 2. Development of drought conditions in drum cans. (A) Structure of drum cans used for the drought experiment. (B) Culture conditions in the drum cans, showing the status of wheat seedlings. (C) Moisture conditions of the top 20 cm of soil inside the drum cans.

Figure 3. Role of KODA for seed germination under drought. Six plants per concentration were sown. Percentages indicate the germination rate as calculated using formula (Materials and methods). Single and double asterisks indicate significant differences at the 5% and 1% level by analysis of variance, respectively. ns, not significant.

Figure 4. Whole plant view of physiologically matured wheat harvested from the drum can. (*Left panel*) Lalmi-2. (*Middle panel*) SR-823. (*Right panel*) LR-744. The photograph shows a representative of four replicates.

Figure 5. Role of KODA in increasing root and shoot length. (A) Root and shoot length balance. (B) Root and shoot biomass balance. Values are means $\pm$ SE (n = 6). Percentages indicate the rate of reduction due to KODA treatment. Single and double asterisks indicate significant differences at the 5% and 1% levels, respectively (ANOVA). N, normal; D, drought.

Fig. 6. Expression of root-specific wheat expansin genes in developing seedling without or with 1 μ M and 5 μ M KODA treatment. (*Left panel*) Time course semi-quantitative RT-PCR analysis of *TaEXPA4* and *TaEXPB10* genes on 1.5% agarose gel stained with ethidium bromide. A wheat Actin used as internal loading control ubiquitously expressed in root and leaf tissues. (*Right panel*) *TaEXPA4* and *TaEXPB10* mRNA expression levels; error bars indicate standard deviations (n = 6). Data are average of technical triplicates and equivalent results were obtained by using duplicate independent biological samples. Percentages indicate the rate of mRNA expression reduction due to KODA treatment. M, 0.1-2kbp DNA marker; w, week.

Figure 7. Model for the presumed mode of KODA action for greater root growth and plant development under CaCO₃ and drought conditions. For further details, see Discussion section.

(A)

Yumeshiho

LR-504

(B)

Fig. 1

(A)

(B)

(C)

Fig. 2

Fig 3.

Fig. 4

Fig. 6.

Fig. 7

Title: Wide recovery ability of wheat from application of α -ketol derivative of linolenic acid, KODA, in response to various abiotic stresses

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Graphical Abstract