

Effects of ZJ0273 on barley and growth recovery of herbicide-stressed seedlings through application of branched-chain amino acids*

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Abstract: In this study, we evaluated the effect of the herbicide propyl 4-(2-(4,6-dimethoxypyrimidin-2-yloxy)benzylamino) benzoate (ZJ0273) on barley growth and explored the potential to trigger growth recovery through the application of branched-chain amino acids (BCAAs). Barley plants were foliar-sprayed with various concentrations of ZJ0273 (100, 500, or 1000 mg/L) at the four-leaf stage. Increasing either the herbicide concentration or measurement time after herbicide treatment significantly impaired plant morphological parameters such as plant height and biomass, and affected physiological indexes, i.e. maximal photochemical efficiency (F_v/F_m), quantum yield of photosystem II (Φ_{PSII}), net photosynthetic rate (P_n), and chlorophyll meter value (soil and plant analyzer development (SPAD)). Cellular injury of herbicide-treated plants was also evidenced by increased levels of reactive oxygen species (ROS) and antioxidative enzyme activity. Elevated levels of herbicide significantly reduced the activity of acetolactate synthase (ALS)—a key enzyme in the biosynthesis of BCAAs. In a separate experiment, growth recovery in herbicide-stressed barley plants was studied using various concentrations of BCAAs (10, 50, 100, and 200 mg/L). Increasing BCAA concentration in growth media significantly increased the biomass of herbicide-stressed barley seedlings, but had no significant effect on non-stressed plants. Further, BCAAs (100 mg/L) significantly down-regulated ROS and consequently antioxidant enzyme levels in herbicide-stressed plants. Our results showed that exogenous application of BCAAs could reverse the inhibitory effects of ZJ0273 by restoring protein biosynthesis in barley seedlings.

Key words: Antioxidant enzyme activity; Barley; Branched-chain amino acid; Photosynthetic system; Reactive oxygen species; ZJ0273

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1 Introduction

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Barley (*Hordeum vulgare* L.) is an important cereal crop widely cultivated for food and forage purposes throughout the world. Weeds such as *Alopoceras aequalis* and *Malachium aquaticum* are the major issue for barley production systems, and can seriously inhibit the growth, yield, and quality of barley crops (Zhang et al., 2000). For example, barley

yields have been reduced by 28.1% in India (Pandey et al., 1998) and 6%–79% in Canada (Watson et al., 2006), due to season-long weed competition. In China, weeds in barley production systems are managed partly by crop rotation with rapeseed. This practice leads to an overlapping of weeds in rapeseed and barley fields. In addition, herbicides such as propyl 4-(2-(4,6-dimethoxypyrimidin-2-yloxy)benzylamino) benzoate (ZJ0273), a broad-spectrum herbicide, are frequently used in oilseed rape production systems in China (Lü et al., 2004; Liu et al., 2008; Yang et al., 2008). Several advantages of this herbicide have been reported such as its high potential for targeting weed plants, low dosage, low mammalian toxicity, broad weeding spectrum and environmental compatibility across Chinese agricultural systems (Chen et al., 2005; Zhang et al., 2009; Jin et al., 2010). Yang et al. (2008) reported an extensive application of ZJ0273 across Chinese rapeseed fields with about 90% eradication efficiency for monocotyledonous and dicotyledonous weeds. This herbicide when applied at the rate of 100 mg/L can effectively reduce *M. aquaticum* and *A. aequalis* in oilseed fields (Zhang et al., 2009). In contrast, higher dosages negatively affect the photosynthetic efficiency of oilseed rape (Jin et al., 2010).

Rapeseed, a dicotyledonous crop, has been found to behave differently from rice, a monocotyledonous crop, in response to ZJ0273 application. Li et al. (2009) found that monocot species can absorb higher amounts of ZJ0273 than dicots at any given sampling time. This was supported by Guo et al. (2011), who observed a significant reduction in the volume and number of vacuoles and the mitotic index in ZJ0273-treated barley cells.

Acetolactate synthase (ALS), an enzyme that catalyzes the first step of parallel reactions in the biosynthesis of branched-chain amino acids (BCAAs) in plants (Leyval et al., 2003), has been found to be the main target of ZJ0273. Previous studies suggested that ZJ0273 arrested weed growth by inhibiting ALS activity (Zhou et al., 2007) and thus BCAA synthesis, leading to impaired protein biosynthesis (Liu et al., 2008; Tian et al., 2014). In plants, changes in the activity of enzymes which catalyze biosynthesis of BCAAs can be reflected in the BCAA content of treated plant leaves (Ortega and Bastide, 1997; Wright et al., 1998). Considering the importance of BCAAs in plant defense mechanisms, we proposed

that exogenously applied BCAAs may restore growth of herbicide-stressed plants. To our knowledge, little information is available on the morphological or physiological adaptation of barley seedlings to stress induced by ZJ0273. In this paper, we elucidate the biochemical pathways through which the herbicide ZJ0273 damages barley growth and explore the potential of BCAAs to promote growth recovery in herbicide-stressed seedlings.

2 Materials and methods

2.1 Plant materials and treatments

Seeds of barley (*Hordeum vulgare* L.) cultivar Zhenongda 3 were obtained from the Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, China. Herbicide ZJ0273 was supplied by the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, China. Field trials were conducted at the Zhejiang University farm, Hangzhou, China (30°10' N, 120°12' E). Conventional crop management was used throughout the growing period. At the four-leaf stage, barley seedlings were foliar-sprayed once with different concentrations of ZJ0273 (0, 100, 500, or 1000 mg/L). Field trials were conducted to explore the effects of the herbicide on plant growth (plant height, biomass), chlorophyll fluorescence (maximal photochemical efficiency (F_v/F_m)), the quantum yield of photosystem II (Φ_{PSII}), and non-photochemical quenching (NPQ)), and physiological attributes (concentrations of ALS, glutathione transferases (GSTs), BCAAs, and total amino acids (TAAs)) of the seedlings. The experiment was arranged in a completely randomized design (CRD) with three replicates.

A hydroponic experiment was conducted to study growth recovery of ZJ0273-stressed barley seedlings following BCAA application. Mature barley seeds were surface-sterilized with 0.15 g/mL NaClO for 15 min and then washed three times with distilled water. The seeds were arrayed in a germination incubator at a temperature of 20 °C under a 16-h photoperiod (light intensity of 140 $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$) and high relative humidity (70%–80%), on a double layer of sterilized filter paper until germination. Seedlings were transplanted into plastic casks containing Hoagland solution, which was continuously aerated with

an air pump. Two-week-old plantlets were exposed to hydroponic solution containing the herbicide ZJ0273 (100 mg/L) or no herbicide and with or without BCCAs (0, 10, 50, 100, or 200 mg/L) for 7 d. Then the growth parameters such as plant height and biomass were calculated to evaluate the mitigation effects contributed by BCAAs.

2.2 Determination of morphological and physiological characteristics

In the field trials, the morphological measurements were conducted at 5, 10, and 15 d after treatment (DAT) to estimate the effects of different concentrations of ZJ0273 (0, 100, 500, or 1000 mg/L) on plant growth. Six plants per treatment were harvested for measuring shoot length and fresh and dry weights. Plant samples were then oven-dried at 110 °C for 0.5 h and then at 80 °C until they reached a constant dry weight (Momoh and Zhou, 2001). In the hydroponic testing, morphological characteristics were also measured to observe the alleviatory effects of BCAAs. All measurements were made in triplicate.

The middle region of the topmost fully expanded barley leaves was used for determining ALS activity following the methods of Yang et al. (2000) and Leyval et al. (2003) with some modifications. GST activity was analyzed according to Irzyk and Fuerst (1993) and Hatton et al. (1996). The absorbance of the mixture was monitored at 340 nm for 120 s. An extinction coefficient of 9.6 L/(mmol·cm) was used to quantify conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH).

Fresh leaf samples (0.2 g) were homogenized in 5 mL potassium phosphate buffer (50 mmol/L, pH 7.8) and then centrifuged at 10000g for 20 min at 4 °C. The supernatant was used for analyzing antioxidant enzyme activity, reactive oxygen species (ROS), and malondialdehyde (MDA) content. The method of Zhou et al. (1997) was used to determine superoxide dismutase (SOD) activity following inhibition of photochemical reduction by nitro blue tetrazolium (NBT). Peroxidase (POD) activity was determined from variation in guaiacol absorbance of the mixture assay at 470 nm (Zhou and Leul, 1998). Catalase (CAT) activity was measured at 240 nm according to Cakmak et al. (1993). Ascorbate peroxide (APX) activity was determined according to Nakano and Asada (1981) with some modifications. Glutathione

reductase (GR) activity was determined according to Jiang and Zhang (2002).

MDA content was measured using a reaction solution (0.5% thiobarbituric acid (TBA) made in 5% trichloroacetic acid (TCA)) (Zhang et al., 2008). The extract mixture in buffer solution was heated at 95 °C for 15 min and then immediately cooled on ice. The samples were centrifuged at 4800g for 10 min and the absorbance of the supernatant was measured at 532 nm. Production of ROS such as H₂O₂ and O₂⁻ was determined according to Al-Aghabary et al. (2004) and Jiang and Zhang (2001), respectively.

The GSH and ascorbic acid (ASA) contents were measured according to Anderson (1985) and Law et al. (1983), respectively. Fresh leaf samples (0.2 g) were homogenized with 0.05 g/mL TCA (5 mL) in an ice bath and centrifuged at 4000g for 10 min. The resultant supernatant was used to measure GSH and ASA contents.

2.3 Photosynthesis and chlorophyll fluorescence

The middle region of the topmost fully expanded leaves was selected for estimating leaf chlorophyll fluorescence using a portable fluorometer (Mini-PAM, Walz, Effeltrich, Germany). The leaves were pre-conditioned in the dark for 30 min, and then minimal fluorescence (F_0) with all photosystem II (PSII) reaction centers opened was determined under weak light (0.04 μmol/(m²·s)). Maximal fluorescence (F_m) with all PSII reaction centers closed was measured with a 0.8-s saturating pulse at 5000 μmol/(m²·s) in dark-adapted leaves. An actinic light (330 μmol/(m²·s)) was applied until these parameters were at steady state with saturating flashes. From each of these, the value of F_m' , the maximum fluorescence in the light, was detected. The steady-state value of fluorescence immediately before a flash of light was termed F_t . $(F_m - F_0)/F_m$ or F_v/F_m was considered as a measure of the maximum quantum yield of PSII in the dark. NPQ was calculated as $(F_m - F_m')/F_m'$. The Φ_{PSII} was defined as $(F_m' - F_t)/F_m'$ (Maxwell and Johnson, 2000). Chlorophyll meter values (soil and plant analyzer development (SPAD) readings) were measured using a chlorophyll meter (Minolta SPAD-502, Japan). The net photosynthetic rate (P_n) of leaves was measured using a portable photosynthesis system (Model Li-6400, USA). Each measurement was replicated three times.

2.4 Determination of endogenous free amino acid content

Fresh leaf samples were collected randomly to measure the concentrations of endogenous free amino acids including three BCCAs (valine (Val), leucine (Leu), and isoleucine (Ile)) and a total of 17 amino acids (TAAs) at three different time intervals, i.e. 5, 10, and 15 DAT. The samples were fixed in an oven at 110 °C for 30 min and then at 80 °C until constant weight. Dried samples were ground into powder and sieved through 60-mesh. Free amino acid content was determined according to the method of Zhu and Zhao (1994). Powder was rubbed in 1 mL of 80% alcohol until volatilization and then homogenized with 1 mL 4% sulfosalicylic acid before extracting using an ultrasonicator for 20 min. The supernatant was collected after centrifugation at 12000g for 15 min and mixed with 1 mL 4% sulfosalicylic acid in deposition, and the extraction procedure was repeated. The two supernatants were merged and sterilized with a 0.25-μm filter. Free amino acid content was calculated by an automatic amino acid analyzer (Hitachi, L-8900, Japan).

2.5 Statistical analysis

In these experiments, three replicates were used for each measurement. Data were analyzed using the Statistical Analysis System (SAS). Significant effects

were determined by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test to identify homogenous groups within the means at $P \leq 0.05$ level.

3 Results

3.1 Changes in free amino acid content

A significant increase in BCAAs such as Val, Leu, and Ile was recorded in response to increasing the ZJ0273 application rate or the treatment duration in this field trial (Table 1). BCAAs in barley leaves remained unaffected by the lower herbicide (100 mg/L) level at 5 DAT, but the treated plants experienced a significant increase in BCAAs at 10 and 15 DAT. No significant change in BCAAs was observed when the ZJ0273 treatment rate was increased beyond 100 mg/L at 10 DAT. BCAAs were undetectable in plant tissues under higher ZJ0273 treatments (500 and 1000 mg/L), when treatment duration was prolonged to 15 d.

TAAs content in plant tissues also increased significantly in response to increasing ZJ0273 concentrations. Higher (500 and 1000 mg/L) ZJ0273 concentrations significantly increased TAAs and decreased BCAAs/TAAs compared with untreated controls at 5 and 10 DAT. Under the prolonged treatment duration (15 DAT) of ZJ0273 (500 and

Table 1 Effects of different concentrations of ZJ0273 on the concentrations of free amino acids and BCAAs/TAAs at the second-leaf stage of barley seedlings

DAT	Concentration of ZJ0273 (mg/L)	Concentration of free amino acids (mg/100 mg DW)				BCAAs/TAAs (%)
		Val	Leu	Ile	TAAs	
5	0	1.816 ^b	1.977 ^b	2.023 ^b	29.450 ^d	19.75 ^a
	100	1.996 ^{ab}	2.231 ^b	2.244 ^b	32.921 ^c	19.66 ^a
	500	1.936 ^{ab}	2.256 ^b	2.318 ^{ab}	37.957 ^b	17.15 ^c
	1000	2.139 ^a	2.908 ^a	2.666 ^a	43.070 ^a	17.91 ^b
10	0	1.653 ^b	2.239 ^b	1.733 ^b	31.065 ^c	18.11 ^b
	100	2.167 ^a	2.660 ^a	2.342 ^a	36.059 ^b	19.88 ^a
	500	2.265 ^a	2.635 ^a	2.561 ^a	40.209 ^b	18.55 ^b
	1000	2.529 ^a	2.842 ^a	2.838 ^a	46.487 ^a	17.66 ^c
15	0	1.865 ^b	2.133 ^b	2.231 ^a	32.403 ^b	19.23 ^a
	100	2.336 ^a	4.376 ^a	2.312 ^a	47.293 ^a	19.08 ^a
	500					
	1000					

Within a column, means at the same DAT followed by the same letters are not significantly different by the LSD test at $P \leq 0.05$ under completely randomized design. Data were analyzed separately at each measurement time. DAT: days after treatment; Val: valine; Leu: leucine; Ile: isoleucine; TAAs: total amino acids; BCAAs: branched-chain amino acids

1000 mg/L), TAAs were not detectable due to the death of the plants, suggesting that longtime treatment duration with higher dosages of ZJ0273 (500 and 1000 mg/L) was toxic for barley seedlings.

3.2 Plant growth response to different concentrations of ZJ0273

Fig. 1 shows the plant growth in response to the different concentrations of ZJ0273 (0–1000 mg/L) at the fourth-leaf stage of barley. Treatments with ZJ0273 (100–1000 mg/L) significantly inhibited plant growth, as reflected by the reductions in plant height, fresh weight, and dry weight of barley seedlings at 5 DAT. Similar trends were observed at 10 and 15 DAT, compared with controls. Plant development in the controls was obviously better than that in all treated plantlets. The growth of barley was obviously suppressed under the stress of 100 mg/L ZJ0273 compared with the control. With the successive increases of ZJ0273 concentrations from 100 to 1000 mg/L, the shoot length and biomass of barley dramatically declined. However, the barley plantlets did not show a significant difference between 500 and 1000 mg/L ZJ0273 for shoot length or biomass at all DAT (Fig. 1). When plants were treated with 1000 mg/L of ZJ0273, 24.9%, 22.2%, and 27.9% reductions in shoot length were observed at 5, 10, and 15 DAT, respectively, compared with controls. Prolonging the DAT from 5 to 15 led to growth recovery under the lower dosage of ZJ0273 (100 mg/L), as reflected by the shoot length, fresh weight, and dry weight. However, these trends were not observed with the application of higher dosages of ZJ0273 (500 and 1000 mg/L). Thus, 100 mg/L ZJ0273 was relatively safe for the barley seedlings.

3.3 Chlorophyll fluorescence and photosynthesis

Increasing ZJ0237 rates of application significantly reduced maximal photochemical efficiency (F_v/F_m) of barley leaves at 1 DAT, and this reduction grew in the subsequent measurements (1–9 DAT; Table 2). The maximum reduction in F_v/F_m was observed in response to 1000 mg/L ZJ0273, which caused a 14.1% reduction (compared with controls) in F_v/F_m at 9 DAT. Changes in leaf Φ_{PSII} in response to ZJ0237 application followed a similar pattern to that of F_v/F_m . Although 100 mg/L ZJ0273 had no significant effect on Φ_{PSII} at 1 DAT, a steep reduction in

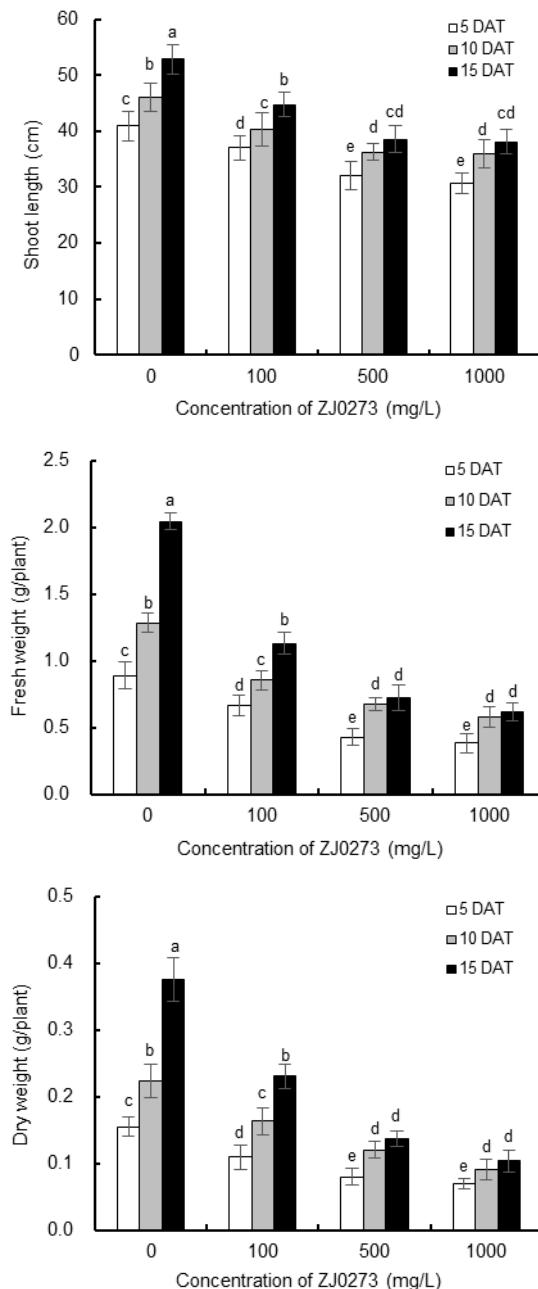


Fig. 1 Effects of different concentrations of ZJ0273 (0–1000 mg/L) on plant height and biomass of barley seedlings

Measurements were made at 5, 10, and 15 d after treatment (DAT). Values are expressed as mean±standard error (SE), $n=3$. Means followed by the same letters did not differ significantly by the LSD test at $P\leq 0.05$.

leaf Φ_{PSII} was recorded under 1000 mg/L ZJ0273 with increasing application period. For example, Φ_{PSII} was reduced by 13.6% at 1 DAT and by 46.4% at 9 DAT (compared with controls). In contrast to F_v/F_m , a

significant increase in NPQ of barley leaves was observed in response to increasing the ZJ0237 application rate or the time after treatment, although the lower herbicide concentration (100 mg/L) had no significant effect on NPQ at 1 DAT. The 1000 mg/L ZJ0273 treatment increased NPQ by 72.8% at 1 DAT and by 124.4% at 9 DAT, compared with controls. No significant differences in the NPQ of the plants were detected between treatments of 500 or 1000 mg/L of ZJ0237 when measured at 5–9 DAT (Table 2).

Table 2 Effects of different concentrations of ZJ0273 on F_v/F_m , Φ_{PSII} , and NPQ in PSII at the fourth-leaf stage of barley seedlings

DAT	Concentration of ZJ0273 (mg/L)	F_v/F_m	Φ_{PSII}	NPQ
1	0	0.816 ^a	0.550 ^a	0.180 ^b
	100	0.808 ^b	0.534 ^a	0.195 ^b
	500	0.805 ^{bc}	0.478 ^b	0.316 ^a
	1000	0.800 ^c	0.475 ^b	0.311 ^a
3	0	0.813 ^a	0.511 ^a	0.185 ^d
	100	0.794 ^b	0.445 ^b	0.283 ^c
	500	0.788 ^b	0.417 ^c	0.397 ^b
	1000	0.785 ^b	0.409 ^c	0.433 ^a
5	0	0.815 ^a	0.517 ^a	0.181 ^c
	100	0.799 ^b	0.421 ^b	0.336 ^b
	500	0.762 ^c	0.364 ^c	0.411 ^a
	1000	0.758 ^c	0.361 ^c	0.435 ^a
7	0	0.816 ^a	0.539 ^a	0.209 ^c
	100	0.780 ^b	0.382 ^b	0.400 ^b
	500	0.750 ^c	0.352 ^c	0.443 ^a
	1000	0.725 ^d	0.338 ^c	0.440 ^a
9	0	0.817 ^a	0.552 ^a	0.205 ^c
	100	0.755 ^b	0.428 ^b	0.427 ^b
	500	0.729 ^c	0.326 ^c	0.448 ^{ab}
	1000	0.702 ^c	0.296 ^d	0.460 ^a

Within a column, means at the same DAT followed by the same letters are not significantly different by the LSD test at $P \leq 0.05$ under completely randomized design. Data were analyzed separately at each measurement time. DAT: days after treatment; F_v/F_m : maximal photochemical efficiency; Φ_{PSII} : quantum yield of PSII; NPQ: non-photochemical quenching

SPAD values of barley leaves declined significantly with each successive increase in the ZJ0273 application rate (Table 3). In addition, reductions in leaf SPAD were observed as the time after treatment was increased. For example, addition of 1000 mg/L ZJ0273 caused reductions in leaf SPAD of 57.7% at 20 DAT and 65.9% at 30 DAT, compared with controls. Damage to leaf greenness resulted in reductions

in leaf P_n under increasing ZJ0273 concentrations and increasing measurement time after treatment. The maximum reduction in leaf P_n was observed under 1000 mg/L ZJ0273, causing a 75.6% reduction compared with the control at 30 DAT (Table 3).

Table 3 Effects of different concentrations of ZJ0273 on the SPAD value and P_n at the fourth-leaf stage of barley seedlings

DAT	Concentration of ZJ0273 (mg/L)	SPAD	P_n
20	0	36.37±0.96 ^a	24.11±1.32 ^a
	100	22.37±1.72 ^b	16.11±1.98 ^b
	500	18.07±1.16 ^c	12.54±0.32 ^c
	1000	15.37±0.71 ^d	6.88±0.92 ^d
30	0	36.90±1.10 ^a	24.54±1.17 ^a
	100	19.90±0.26 ^b	12.69±0.34 ^b
	500	15.70±1.00 ^c	9.49±0.77 ^c
	1000	12.57±1.39 ^d	6.00±0.38 ^d

Within a column, means at the same DAT followed by the same letters are not significantly different by the LSD test at $P \leq 0.05$ under completely randomized design. Data were analyzed separately at each measurement time. Values are expressed as mean±SE ($n=3$). DAT: days after treatment; SPAD: chlorophyll meter value (soil and plant analyzer development); P_n : net photosynthetic rate

3.4 Endogenous enzyme activity

No significant effect of the lower ZJ0273 concentration (100 mg/L) was observed on ALS activity of barley leaves at 5 or 10 DAT (Table 4), although higher application rates significantly reduced ALS activity. ALS activity was significantly reduced in response to all ZJ0273 application rates when measured at 15 and 20 DAT. With successive increases in ZJ0273 concentrations from 100 to 1000 mg/L, ALS activity dramatically decreased. When plants were treated with 1000 mg/L of ZJ0273, the ALS activity of barley leaves was reduced by 28.3% at 5 DAT, 36.3% at 10 DAT, 50.2% at 15 DAT, and 57.7% at 20 DAT compared with controls.

GST activity of barley leaves increased in response to increasing herbicide application rates, although no significant change in GST activity was observed by increasing ZJ0273 concentrations from 500 to 1000 mg/L at 5 or 10 DAT (Table 4). Maximum GST activity (20.90 nmol/(min·mg protein)) was observed under 1000 mg/L ZJ0273 at 20 DAT, when it was 215.7% greater than that of the control leaves.

Table 4 Effects of different concentrations of ZJ0273 on ALS and GST activity at the fourth-leaf stage of barley seedlings

DAT	ZJ0273 (mg/L)	ALS activity (OD ₅₃₀ /(g FW·min))	GST activity (nmol/(min·mg protein))
5	0	1.87±0.16 ^a	5.85±0.31 ^c
	100	1.83±0.36 ^a	7.76±0.76 ^b
	500	1.62±0.41 ^b	11.30±0.81 ^a
	1000	1.34±0.35 ^c	11.83±1.48 ^a
10	0	1.90±0.45 ^a	6.61±0.53 ^b
	100	1.81±0.42 ^a	8.05±0.66 ^b
	500	1.59±0.24 ^b	12.84±1.07 ^a
	1000	1.21±0.52 ^c	14.04±2.14 ^a
15	0	2.07±0.21 ^a	6.59±0.50 ^d
	100	1.69±0.19 ^b	10.41±0.61 ^c
	500	1.52±0.38 ^c	13.86±1.12 ^b
	1000	1.03±0.41 ^d	16.05±0.86 ^a
20	0	2.15±0.22 ^a	6.62±0.46 ^d
	100	1.65±0.25 ^b	15.40±1.04 ^c
	500	1.47±0.19 ^c	17.93±0.94 ^b
	1000	0.91±0.17 ^d	20.90±1.61 ^a

Within a column, means at the same DAT followed by the same letters are not significantly different by the LSD test at $P\leq 0.05$ under completely randomized design. Data were analyzed separately at each measurement time. Values are expressed as mean±SE ($n=3$). DAT: days after treatment; ALS: acetolactate synthase; GST: glutathione transferase; OD₅₃₀: optical density at 530 nm; FW: fresh weight

3.5 Growth of ZJ0273-stressed seedlings in response to exogenously applied branched-chain amino acids

A significant reduction in shoot length, fresh and dry weights was recorded in barley seedlings in response to 100 mg/L of ZJ0273 (Table 5). Plants treated with 100 mg/L ZJ0273 alone experienced maximum damage in this hydroponic study, showing reductions of 31.3% in shoot length, 30.2% in fresh weight, and 25.0% in dry weight, compared with controls. Exogenously applied BCAAs (10–200 mg/L) in the nutrient solution significantly increased the growth of barley seedlings in terms of shoot length and fresh and dry weights. Application of BCAAs to herbicide-free media also promoted growth of barley seedlings, but the effect on shoot length or dry weight was not significant compared with the control (non-BCAA, non-ZJ0273 treated). Maximum growth (shoot length, fresh

and dry weights) of herbicide-stressed seedlings was observed with 200 mg/L of BCAAs. Plants treated with 200 mg/L BCAAs alone showed no significant change in growth. BCAAs significantly promoted barley growth when applied to ZJ0273 (100 mg/L)-stressed plants, causing increases of 37.0% in shoot length, 38.1% in fresh weight, and 33.9% in dry weight, compared with the 100 mg/L ZJ0273 treatment alone. The fresh and dry weights of the plants remained unaffected when treated with 100 mg/L ZJ0273+200 mg/L BCAAs. No significant changes occurred in the shoot growth of stressed seedlings with 100 or 200 mg/L of BCAAs, suggesting that 100 mg/L of BCAAs could be the best concentration for protecting barley seedlings from herbicide stress. Thus, we selected this concentration for the subsequent physiology experiments.

Table 5 Effects of exogenous BCAAs on the shoot length, fresh weight, and dry weight of barley seedlings treated with 100 mg/L ZJ0273

Concentration (mg/L)	Shoot length (cm)	Fresh weight (g/10 plants)	Dry weight (g/10 plants)
Z0	19.40 ^a	2.186 ^{bcd}	0.220 ^{abc}
Z0+aa10	19.46 ^a	2.223 ^{bc}	0.223 ^{ab}
Z0+aa50	19.49 ^a	2.338 ^{ab}	0.230 ^a
Z0+aa100	19.51 ^a	2.398 ^a	0.239 ^a
Z0+aa200	19.77 ^a	2.469 ^a	0.236 ^a
Z100	13.32 ^e	1.526 ^f	0.165 ^e
Z100+aa10	14.78 ^d	1.715 ^e	0.191 ^d
Z100+aa50	16.03 ^c	1.814 ^e	0.200 ^{cd}
Z100+aa100	18.08 ^b	2.050 ^d	0.210 ^{bcd}
Z100+aa200	18.25 ^b	2.108 ^{cd}	0.221 ^{abc}

Within a column, means followed by the same letters are not significantly different by the LSD test at $P\leq 0.05$ under completely randomized design. Z0: control; Z100: 100 mg/L ZJ0273; aa10: 10 mg/L BCAAs (valine, leucine, isoleucine); aa50: 50 mg/L BCAAs; aa100: 100 mg/L BCAAs; aa200: 200 mg/L BCAAs

ZJ0273 treatment (100 mg/L) increased the generation of ROS such as O₂⁻ and H₂O₂ in barley leaf tissues (Fig. 2). In contrast, addition of 100 mg/L BCAAs to the solution significantly decreased ROS generation in herbicide-treated seedlings. BCAA application caused reductions of 36.4% in O₂⁻ and 24.4% in H₂O₂ content of herbicide-stressed plants, compared with ZJ0273 treatment alone. BCAAs applied to herbicide-free media had no significant effect on leaf O₂⁻ or H₂O₂ content.

ASA and GSH content in leaf tissues increased significantly in response to 100 mg/L ZJ0273 and decreased when BCAAs were added to the growth media. BCAAs caused reductions of 20.2% in ASA content and 10.1% in GSH content of the leaves of herbicide-treated seedlings, compared with ZJ0273 treatment alone. The effect of BCAAs on herbicide-free seedlings was non-significant (Fig. 3).

The activity of various antioxidant enzymes (POD, SOD, CAT, APX, and GR) and leaf peroxidation (MDA) increased significantly in response to ZJ0273 treatment (Fig. 4). BCAAs applied exogenously to

herbicide-stressed seedlings, significantly reduced antioxidant enzyme activity and MDA content. For example, BCAAs applied to herbicide-stressed plants decreased the activity of POD by 19.4%, SOD by 35.3%, CAT by 17.2%, and APX by 48.6%, compared with ZJ0273 treatment alone, although GR activity remained unaffected. No significant effect of BCAA application was observed on antioxidant enzymes or the MDA content of barley seedlings in herbicide-free media, except for SOD and APX activity, which increased significantly under BCAA treatment under both herbicide-stressed and herbicide-free conditions.

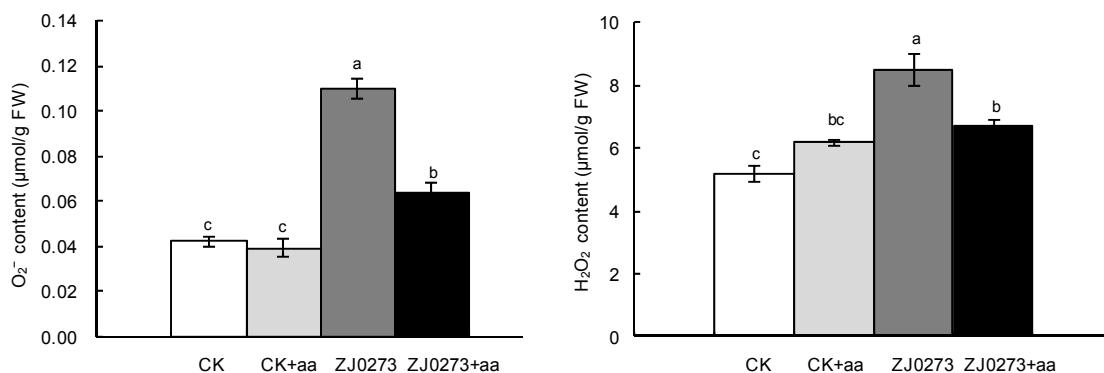


Fig. 2 Effects of exogenous BCAAs (100 mg/L) on ROS (O_2^- or H_2O_2) concentrations in barley seedlings treated with 100 mg/L ZJ0273

Values are expressed as mean \pm SE ($n=3$). Means followed by the same letters did not differ significantly by the LSD test at $P\leq 0.05$. CK: control, no ZJ0273; aa: BCAAs; FW: fresh weight

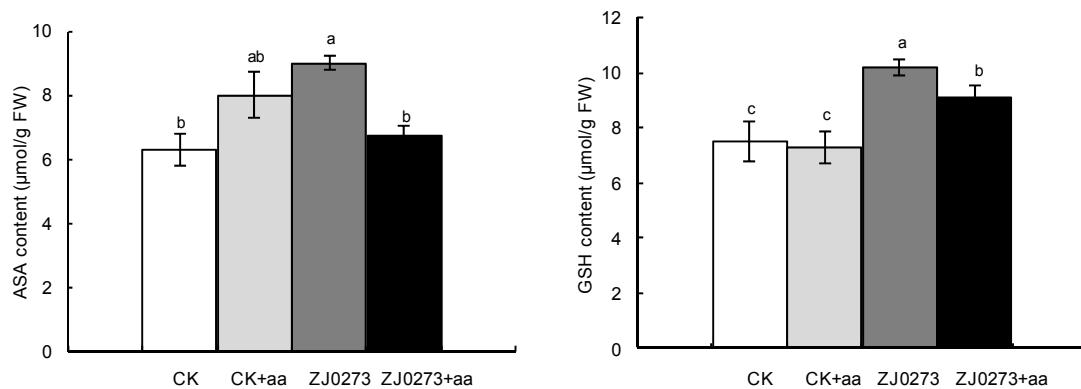


Fig. 3 Effects of exogenous BCAAs (100 mg/L) on ascorbic acid (ASA) and glutathione (GSH) concentrations in barley seedlings treated with 100 mg/L ZJ0273

Values are expressed as mean \pm SE ($n=3$). Means followed by the same letters did not significantly differ by the LSD test at $P\leq 0.05$. CK: control, no ZJ0273; aa: BCAAs; FW: fresh weight

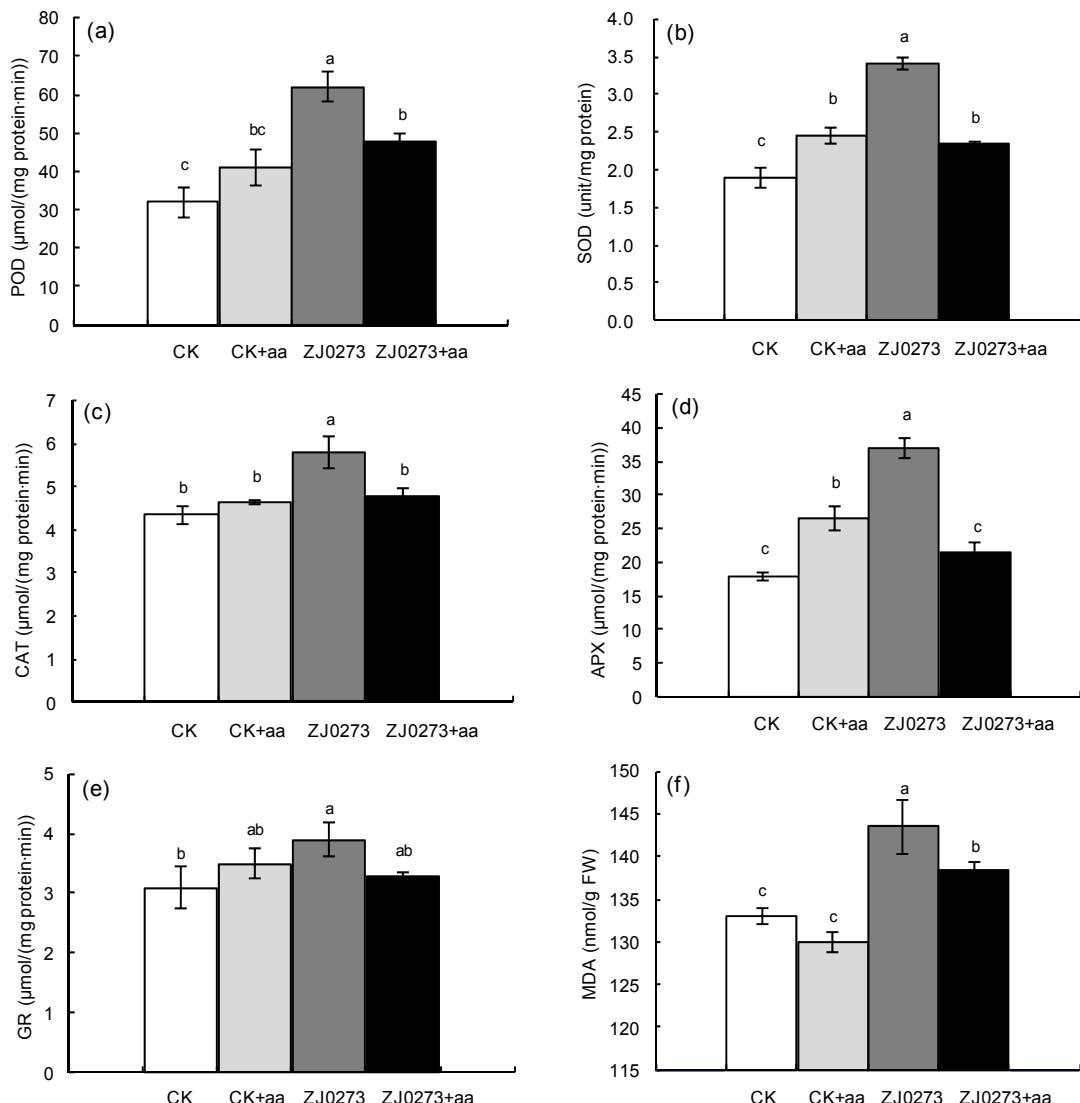


Fig. 4 Effects of exogenous BCAAs (100 mg/L) on biochemical changes in barley seedlings treated with 100 mg/L ZJ0273

(a) Peroxidase (POD) activity; (b) Superoxide dismutase (SOD) activity; (c) Catalase (CAT) activity; (d) Ascorbate peroxide (APX) activity; (e) Glutathione reductase (GR) activity; (f) Malondialdehyde (MDA) content. Values are expressed as mean±SE ($n=3$). Means followed by the same letters did not significantly differ by the LSD test at $P\leq 0.05$. CK: control, no ZJ0273; aa: BCAAs; FW: fresh weight

4 Discussion

The first objective of these experiments was to identify the mechanism by which herbicide ZJ0273 inhibits barley growth. A significant reduction in SPAD and chlorophyll fluorescence of herbicide-stressed leaves indicated that ZJ0273 can impair chlorophyll structure and function. Ralph et al. (2005) indicated that inhibition curves of chlorophyll

fluorescence parameters varied with different herbicide concentrations. Damage to photosynthetic machinery leads to an overall inhibition of photosynthesis and biomass production (Anderson, 1985; Cobb and Kirkwood, 2000; Abbaspoor et al., 2006; Ali et al., 2018).

Various biochemical pathways for stress-induced P_n reduction have been proposed. The interrupted biosynthesis of proteins associated with the carbon assimilation process has been suggested by Halliwell

and Gutteridge (1999) and Romero-Puertas et al. (2004). Similarly, Orcaray et al. (2010) indicated that glyphosate can inhibit biosynthesis of aromatic amino acids by blocking the shikimate pathway and arresting the photosynthetic process in leaf tissues. Herbicide-induced chlorophyll impairment in this study could have been associated with an increased production of ROS and lipid membrane damage (MDA production), which are the major causes of cellular injury in plants. In contrast to a significant recovery in leaf P_n at 28 DAT in 100 and 500 mg/L ZJ0273-treated *Brassica* plants (Jin et al., 2010), no photosynthesis recovery in barley seedlings suggested a relatively high herbicide sensitivity for monocotyledons. Although stressed barley plants up-regulated defense-related primary antioxidants (POD, SOD, CAT, APX, and GR) and secondary antioxidants (ASA and GSH), no significant growth recovery was observed in this study. This suggests that ZJ0273 can cause permanent cellular damage in barley seedlings and the plants are unable to mitigate this damage through internal antioxidative defense mechanisms. Up-regulation of antioxidant enzymes has also been linked with increased cellular injury to ZJ0273-treated *Brassica* plants (Law et al., 1983; Dixon et al., 1998; Frankart et al., 2003; Jin et al., 2010). Studies of the key enzymes in the biosynthesis of natural products have been important in revealing the mechanism pathways (Yang et al., 2012; Liang et al., 2013; Xia et al., 2015, 2016; Zhang et al., 2017).

Herbicide-induced growth suppression in barley seedlings in this study was associated with inhibited activity of ALS, an enzyme associated with BCAA production. Previous studies indicated that ALS inhibitors can block the biosynthesis of BCAs and cell division, and eventually cause plant death (Ray, 1984; Shaner and Singh, 1993). Chen et al. (2005) suggested that ZJ0273 can inhibit ALS activity by altering the biosynthesis of amino acids. Similarly, using near-infrared spectroscopy (NIRS) techniques, Tian et al. (2014) confirmed a gradual reduction in free amino acids (BCAs and TAAs) and ALS activity in oilseed rape leaves in response to increasing ZJ0273 concentrations. Growth inhibition in ALS inhibitor-treated plants has been linked with a dramatic decrease in specific BCAs (Rost and Reynolds, 1985; Hofgen et al., 1995). In contrast, a significant increase in BCAA content in this study under herbicide application could be the result of protein transformation

to satisfy new protein requirements (Royuela et al., 2000; Orcaray et al., 2010). Similarly, Royuela et al. (2000) and Liu et al. (2008) suggested an increase in BCAA content in response to herbicide application as a result of degradation of some proteins to maintain the balance of new protein synthesis. A significant increase in BCAs/TAAs of ZJ0273-stressed barley leaves in this study supports the suggestion that amino acid composition and thus protein biosynthesis had been altered.

The second objective of this study was to examine the growth recovery of herbicide-stressed barley seedlings following BCAA application. A significant growth recovery in ZJ0273-stressed barley was achieved in response to BCAA application. No significant effect of BCAs on unstressed plants suggested that the primary role of these amino acids was to restore growth of stressed plants without affecting normal plant growth patterns. Exogenously applied amino acids had already been found to be effective in alleviating the negative effects of ALS inhibitors in pea (Rost and Reynolds, 1985) and corn (Shaner and Reider, 1986). Therefore, the herbicide ZJ0273 may block the biosynthetic pathway of BCAs by inhibiting ALS, and exogenous application of BCAs may compensate for the shortage of BCAs induced by the herbicide (Tang et al., 2006; Xia et al., 2006).

5 Conclusions

In this study, we confirmed that tolerance to herbicide increased with the growth of barley. The lower dosage (100 mg/L) of ZJ0273 was safer for barley than the higher dosages (500 and 1000 mg/L). The inhibitory effects of herbicide ZJ0273 on the photosystem and enzymes might be related to the metabolic mechanism of this herbicide. Based on our observations, the lower dosage of ZJ0273 (100 mg/L) could be recommended for weed eradication purposes in barley fields. This study also explored the prospects of using BCAs for improving the growth of barley under herbicide stress.

Contributors

Ling XU, Ullah NAJEEB, and Wei-jun ZHOU designed and conceived the study. Ling XU, Jian-yao SHOU, and Xiang GUO performed the experiments. Ling XU, Jian-yao SHOU, Rafaqat Ali GILL, and Ullah NAJEEB analyzed the data and

wrote the manuscript. All authors revised and approved the paper.

Compliance with ethics guidelines

Ling XU, Jian-yao SHOU, Rafaqat Ali GILL, Xiang GUO, Ullah NAJEEB, and Wei-jun ZHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文摘要

题 目:除草剂丙酯草醚对大麦的影响和支链氨基酸对其生长的恢复作用

目 的:评价除草剂丙酯草醚对大麦生长的影响，探讨外源添加支链氨基酸对其生长的恢复作用。

创新点:从除草剂丙酯草醚作用机理出发，阐明了外源添加支链氨基酸对丙酯草醚作用下大麦各项生理和生长指标的缓解作用，为生长调节物质的进一步开发利用提供实验依据。

方 法:采用水培和大田实验相结合的方法，研究了除草剂丙酯草醚对大麦生长的影响，并通过进一步测定大麦幼苗各项生理指标和支链氨基酸浓度，表明 100 mg/L 支链氨基酸可显著缓解丙酯草醚对大麦的生理损伤，从而恢复其生长。

结 论:外源添加支链氨基酸能够缓解除草剂丙酯草醚对大麦生长的抑制作用。

关键词:抗氧化酶活性；大麦；支链氨基酸；光合系统；活性氧；丙酯草醚