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Alleviation of Waterlogging Damage in Winter Rape by Uniconazole Application: Effects on Enzyme Activity, Lipid Peroxidation, and Membrane Integrity

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Abstract. Oilseed rape (*Brassica napus* L.) seedlings treated with uniconazole [(*E*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-l-yl)-l-penten-3-ol] were transplanted at the five-leaf stage into specially designed experimental containers and then exposed to waterlogging for 3 weeks. After waterlogging stress, uniconazole-treated seedlings had significantly higher activities of superoxide dismutase, catalase, and peroxidase enzymes and endogenous free proline content at both the seedling and flowering stages. Uniconazole plus waterlogging-treated plants had a significantly higher content of unsaturated fatty acids than the waterlogged plants. There was a parallel increase in the lipid peroxidation level and electrolyte leakage rate from the leaves of waterlogged plants. Leaves from uniconazole plus waterlogging-treated plants had a significantly lower lipid peroxidation level and electrolyte leakage rate compared with waterlogged plants at both the seedling and flowering stages. Pretreatment of seedlings with uniconazole could effectively delay stress-induced degradation of chlorophyll and reduction of root oxidizability. Uniconazole did not alter the soluble sugar content of leaves and stems, after waterlogging of seedlings. Uniconazole improved waterlogged plant performance and increased seed yield, possibly because of improved antioxidation defense mechanisms, and it retarded lipid peroxidation and membrane deterioration of plants.

Key Words. Waterlogging—Uniconazole—*Brassica napus* L.—Enzymes—Lipid peroxidation—Membrane integrity

Oilseed rape (*Brassica napus* L.) is one of the world's major oilseed crops and the most important source of edible oil in China. It is expanding rapidly as a rotation crop following rice. Waterlogging is an important global crop production constraint causing significant yield reduction in winter rape, especially when the water table remains near the soil surface after transplanting and establishment (Zhou 1994). Lack of O_2 may limit crop growth because of alterations in metabolism (Drew 1992). A decline in nutrient uptake and the build-up of toxic compounds in the soil have also been reported (Jackson and Drew 1984).

The adverse effects of waterlogging on terrestrial plants are complex and vary with genotype, pretreatment, plant development stage, and the duration and severity of waterlogging. Depending on the climate and the stage of development, significant yield reduction can occur if rape plants are exposed to waterlogging from 3 to 30 days (Gutierrez Boem et al. 1996, Zhou and Lin 1995). Waterlogging leads to senescence by inactivation of antioxidant enzymes, generation of active oxygen species, chlorophyll degradation, lipid peroxidation, and membrane deterioration (Yan et al. 1996, Zhou and Lin 1995). These toxic oxygen species react with numerous cell components, producing a cascade of oxidative reactions that inactivate enzymes and cause lipid peroxidation, protein degradation, DNA strand breakage, and pigment bleaching (Scandalios 1993).

Plant growth regulators play an important role in crop production and are being used increasingly to manipulate plant growth and yield. Uniconazole [(*E*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-l-yl)-1-penten-3-ol], a potent and active member of the triazole family,

Abbreviations: CK, control; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; MDA, malondialdehyde; IUFA, index of unsaturated fatty acids; NBT, nitro blue tetrazolium; REC, relative electrical conductivity; TTC, triphenyl tetrazolium chloride.

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was developed for use as a plant growth retardant (Fletcher et al. 1986). Uniconazole increases the activities of antioxidant enzymes (such as superoxide dismutase and catalase), chlorophyll content, and improves root growth in winter rape (Zhou and Ye 1996). In addition, it enhances nitrate reductase activity and the plant photosynthetic rate and increases soluble protein and the total sugar content (Yang et al. 1994). Recent research indicated that paclobutrazol, a closely related triazole, and Mixtalol could alleviate waterlogging damage in wheat and oilseed rape plants (Webb and Fletcher 1996, Zhou et al. 1997b).

The present investigation was carried out to determine if uniconazole confers waterlogging tolerance to winter rape and if such tolerance is correlated with changes in enzyme activity, lipid peroxidation, and membrane integrity.

Materials and Methods

Plants and Treatments

The experiment was conducted at Zhejiang Agricultural University (Hangzhou, 30°10′N, 120°12′E) during the 1996–1997 season and was based on the preliminary results obtained with uniconazole during the 1995–1996 season. A 5% water-dispersible powder of uniconazole (high effect triazole) was produced and provided by the United Chemical Factory of Zhangjiegang City, Jiangsu Province. Oilseed rape (*B. napus* L. cv. 601) was sown on October 3 on a seed bed of silt loam. 50 mg/liter uniconazole (distilled water as control) was uniformly applied as a foliar spray using a portable sprayer on October 29 (at the three-leaf stage) at the rate of 750 liters of formulated solution/ha. The concentration of 50 mg/liter uniconazole and its application stage were used as in previous investigations (Ye et al. 1995, Zhou and Ye 1996).

12 concrete containers (400 × 100 × 120 cm) with drainage holes 15 cm apart in the side walls were spaced in the field 50 cm apart. Fitted frames were covered with plastic film to prevent entry of rain, as necessary. The containers were filled with a silt loam containing 0.18% total nitrogen, 2.11% organic matter, 52 mg/liter readily available phosphorus ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}), and 127 mg/liter readily available potassium (K^+). Fertilizers, at the rate of 200 kg of nitrogen/ha, 60 kg of P_2O_5/ha, and 110 kg of K_2O/ha, were mixed thoroughly in the soil of each container.

50 seedlings were transplanted at the five-leaf stage on November 6 into each experimental container. 50 days after the seedling transplant, plants were waterlogged to the soil surface for 3 weeks (starting from December 25) in a randomized complete block design in three replicates. In the control (CK), the soil moisture was kept at 80% of field capacity during the entire experiment. Conventional methods of crop management, such as weeding and disease and insect control, were followed during the growing period. After the analysis of variances, statistical inferences were made based on the Duncan's new multiple range test between the different treatment means.

Biochemical Measurement Techniques

Samples for analyses were taken from plants in the central rows of each container at the seedling stage (the end of waterlogging treatment) and at the beginning of the flowering stage. Three plants were sampled for each analytical measurement, and three measurements of each parameter were taken. All of the analyses were performed on the third and fourth leaves from the top, except for malondialdehyde (MDA) which was taken from the first green leaf from the base of the plant.

Leaf superoxide dismutase (SOD) activity was analyzed by the method of Zhu et al. (1990). Leaf tissue (500 mg) was homogenized at 4°C in 5 mL of 50 mM phosphate buffer (pH 7.0) containing 1% insoluble polyvinylpyrrolidone with a mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 10 min, and the supernatant obtained was used as the enzyme extract. SOD activity was assayed essentially as described by Dhindsa et al. (1981) by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The 4-mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 77.12 μM NBT, 0.1 mM EDTA, 13.37 mM methionine, 0–10 μL of enzyme extract, and 100 μL of 80.2 μM riboflavin (riboflavin was added last). Leaf catalase (CAT) activity was analyzed by the H_2O_2 reduction method (Shandong Agricultural College 1980). Leaf extracts were treated with 5 mL of 0.1 N H_2O_2 and kept at 20°C for 5 min. Then 1 mL of a 20% KI solution was added with 3 drops of 10% $(NH_4)_6Mo_7O_{24}$ solution and 5 drops of 1% starch solution. A 0.02 N $Na_2S_2O_3$ reagent was used to titrate the reaction solution until the disappearance of blue color. CAT activity was assayed by determining the rate of reduction of H_2O_2 in a given period. Leaf peroxidase (POD) activity was analyzed by the guaiacol reduction method (Zhang 1992). Leaf enzyme extracts (1 mL) were treated with 1 mL of acetate buffer (pH 5.0) and 1 mL of 0.1% guaiacol and kept at 30°C for 5 min. Then 1 mL of 0.08% H_2O_2 solution was added; 2 min later the extract was read at 470 nm.

Leaf samples were analyzed for fatty acid composition using gas chromatography (GC-9A, Shimadzu, Japan), as described previously (Zhou et al. 1997a).

The leaf proline content was determined using a colorimetric method (Zhang 1992). Leaf samples were treated with 5 mL of 3% sulfosalicylic acid and kept at 100°C for 10 min. The supernatant (1 mL) was added with 2 mL of distilled water, 2 mL of glacial acetic acid, and 4 mL of 2.5% acidic ninhydrin and kept at 100°C for 60 min. The absorbance of the extract was read at 520 nm. MDA was measured as thiobarbituric acid-reactive material from centrifuged leaf extracts in 10% trichloroacetic acid (Dong et al. 1983). The absorbance of the extract was read at 532 nm, and the values were corrected by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using its extinction coefficient. The membrane permeability of a leaf was measured by electrical conductivity (Zhu et al. 1990). The washed leaves (500 mg) were cut into 0.5-cm pieces and placed in a 25-mL test tube containing 15 mL of deionized water. The leaf samples were immersed and vibrated for 30 min, and then the conductivity of the solution was measured using a conductivity meter (DDS-11A). After boiling the samples for 10 min, their conductivity was measured again when the solution was cooled to room temperature. The relative electrical conductivity (REC) was calculated as follows,

$$\mathrm{REC} = \mathrm{C1/C2} \times 100$$

where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

The leaf chlorophyll content was determined by the acetone/ethanol mixture assay method (Chen 1984). Root oxidizability was measured by the triphenyl tetrazolium chloride (TTC) reduction method (Shen et al. 1991). The roots were washed free of soil, and then 500 mg of roots from each treatment was placed in a 25-mL test tube and sealed with a rubber stopper. The tubes were treated with 5 mL of 0.4% TTC solution and 5 mL of 1/15 M phosphate buffer (pH 7.0). After incubation for 3 h at 40°C, the tubes were treated with 2 mL of 2 N H_2SO_4. Then roots were ground with ethyl acetate (a total of 10 mL) to extract red triphenylformazane, and the extract was read at 485 nm.

The plant-soluble sugar content was determined by the an-

Table 1. Effects of uniconazole and waterlogging on the activities of SOD (unit/g FW/min), CAT (H_2O_2 mg/g FW/min), and POD (unit/g FW/min) enzymes of rape leaves.

	Seedling stage			Flowering stage		
Treatment[a]	SOD	CAT	POD	SOD	CAT	POD
CK	36.42 bB[b]	3.79 cB	11.42 bB	68.15 bB	1.63 cB	6.50 bB
Unic	48.35 aA	5.05 aA	16.00 aA	97.78 aA	2.16 aA	10.25 aA
WL	21.81 cC	2.77 dC	7.42 cC	40.28 cC	1.23 dC	4.00 cC
Unic + WL	39.92 bB	4.45 bAB	16.50 aA	74.81 bB	1.94 bA	9.50 aA

[a] CK, control; Unic, foliar application of 50 mg/liter uniconazole on rape seedlings at the three-leaf stage at the rate of 750 liters of formulated solution/ha; WL, waterlogging for 3 weeks at the seedling stage; Unic + WL, uniconazole plus waterlogging treatment.
[b] Within columns, means followed by the same small or capital letters are not significantly different at the 0.05 and 0.01 levels of probability, respectively.

Table 2. Effects of uniconazole and waterlogging on the fatty acid composition (percent of total fatty acids) of rape leaves at the seedling stage.

Treatment[a]	$C_{16:0}$[b]	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	IUFA
CK	8.41 NS[c]	0.80 abAB[d]	12.33 aA	7.40 aA	46.65 aA	167.10 aA
Unic	8.55	0.67 bB	12.45 aA	7.39 aA	47.25 aA	169.00 aA
WL	8.82	0.93 aA	10.56 bB	6.19 bA	43.00 bA	151.90 bA
Unic + WL	8.18	0.74 bAB	12.53 aA	7.80 aA	47.22 aA	169.80 aA

[a] Treatment description is given in Table 1.
[b] $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid; IUFA $= [C_{18:1} + C_{18:2} \times 2 + C_{18:3} \times 3] \times 100$.
[c] NS, not significant at the 0.05 level of probability.
[d] Within columns, means followed by the same small or capital letters are not significantly different at the 0.05 and 0.01 levels of probability, respectively.

throne/ethyl acetate mixture assay method (Zhang 1992). Seed yield (at maturity) was obtained as an aggregate of individual plants.

Results

Foliar sprays of uniconazole significantly increased the activities of SOD, CAT, and POD enzymes of rape leaves compared with the control by 32.8, 33.3, and 40.1% at the seedling stage and by 43.5, 32.5, and 57.7% at the flowering stage, respectively (Table 1). However, waterlogging treatment significantly decreased the leaf SOD, CAT, and POD activities compared with the control by 40.2, 26.9, and 35.0% at the seedling stage and by 40.9, 24.5, and 38.5% at the flowering stage, respectively. There was no apparent difference in the activity of leaf SOD enzyme between the uniconazole plus waterlogging-treated plants and the control at both stages, but the activities of CAT and POD enzymes were significantly higher than the control by 17.4 and 44.5% at the seedling stage and by 19.0 and 46.2% at the flowering stage, respectively.

After exposure of rape seedlings to waterlogging for 3 weeks, the oleic, linoleic, and linolenic acid contents of rape leaves were decreased significantly compared with the control by 14.7, 16.4, and 5.9% at the seedling stage and by 5.2, 7.6, and 5.2% at the flowering stage, respectively (Tables 2 and 3). There was no difference in the fatty acid composition of the leaves between the control and the uniconazole plus waterlogging-treated plants at the seedling or flowering stages. The leaves of the uniconazole plus waterlogging-treated plants had a significantly higher content of oleic, linoleic, and linolenic acids of 18.7, 26.0, and 9.8% at the seedling stage and 7.6, 6.6, and 8.7% at the flowering stage, respectively, over the waterlogged plants. At the seedling stage, the stearic acid content of leaves of uniconazole plus waterlogging-treated plants was significantly lower than the waterlogged plants by 20.4%. The index of unsaturated fatty acids of leaves of uniconazole plus waterlogging-treated plants was similar to the control but significantly higher than the waterlogged plants by 11.8 and 8.4% at the seedling and flowering stages, respectively (Tables 2 and 3).

The endogenous free proline content of the leaf was increased significantly by uniconazole by 22.5 and 20.1% but was decreased by waterlogging by 21.1 and 17.0% compared with the control at the seedling and flowering stages, respectively (Table 4). The leaf proline content of the uniconazole plus waterlogging treatments was significantly higher than the control by 11.3 and

Table 3. Effects of uniconazole and waterlogging on the fatty acid composition (percent of total fatty acids) of rape leaves at the beginning of flowering.

Treatment[a]	$C_{16:0}$[b]	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	IUFA
CK	7.20 NS[c]	0.89 NS	14.50 aA[d]	6.43 bB	43.91 aAB	159.10 aA
Unic	7.24	0.92	14.52 aA	7.30 aA	44.76 aA	163.40 aA
WL	7.45	0.91	13.75 bB	5.94 cB	41.63 bB	150.52 bB
Unic + WL	7.10	0.82	14.79 aA	6.33 bB	45.26 aA	163.23 aA

[a] Treatment description is given in Table 1.
[b] $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid; IUFA, $= [C_{18:1} + C_{18:2} \times 2 + C_{18:3} \times 3] \times 100$.
[c] NS, not significant.
[d] Within columns, means followed by the same small or capital letters are not significantly different at 0.05 and 0.01 level of probability, respectively.

Table 4. Effects of uniconazole and waterlogging on free proline (μg/g FW) and MDA (μmol/g FW) content and the relative electrolyte conductivity (%) of rape leaves.

	Seedling stage			Flowering stage		
Treatment[a]	Proline	MDA	REC	Proline	MDA	REC
CK	129.35 cB[b]	10.17 bB	14.24 cB	22.89 cB	10.88 bB	15.12 bB
Unic	158.51 aA	7.31 dC	11.15 dC	27.49 aA	8.73 dD	11.54 dD
WL	102.07 dC	12.73 aA	18.71 aA	19.00 dC	13.89 aA	18.10 aA
Unic + WL	143.93 bAB	8.00 cC	15.79 bB	25.87 bA	9.48 cC	13.65 cC

[a] Treatment description is given in Table 1.
[b] Within columns, means followed by the same small or capital letters are not significantly different at the 0.05 and 0.01 levels of probability, respectively.

13.0% at the seedling and flowering stages, respectively. Uniconazole significantly reduced the MDA content and electrolyte leakage from leaves compared with the control at both seedling and flowering stages. In contrast, the MDA content and electrolyte leakage from leaves of waterlogged plants were significantly higher than the control by 25.2 and 31.4% and by 27.7 and 19.7% at the seedling and flowering stages, respectively. The MDA content and electrolyte leakage from leaves of uniconazole plus waterlogging-treated plants were significantly lower than the control by 21.3 and 10.9% and by 12.9 and 9.7%, at the seedling and flowering stages, respectively.

The leaf chlorophyll content and root oxidizability of waterlogged plants were reduced significantly by 13.3 and 24.0% and by 16.7 and 21.5% compared with the control at the seedling and flowering stages, respectively (Table 5). The leaf chlorophyll content and root oxidizability of the uniconazole plus waterlogging-treated plants were significantly higher than the control by 15.8 and 16.0% and by 9.0 and 12.3% at the seedling and flowering stages, respectively.

The waterlogged plants had a significantly higher soluble leaf sugar content, 81.6 and 100.8%, over the control at the seedling and flowering stages, respectively (Table 6). At the seedling stage, the soluble stem sugar content of the waterlogged plants was increased significantly by 43.2%, but at the flowering stage it was decreased by 27.2% compared with the control. A significant pretreatment effect of seedlings with uniconazole on the soluble sugar content after exposure of the seedlings to waterlogging was obtained only for the stem-soluble sugar content at the flowering stage, where it was reduced by 6.8% compared with the waterlogging treatment. Uniconazole plus waterlogging-treated plants had a significantly higher seed yield over both the waterlogged plants and the control.

Table 5. Effects of uniconazole and waterlogging on leaf chlorophyll content (mg/g FW) and root oxidizability (TTC mg/g FW/h) of rape plants.

	Seedling stage		Flowering stage	
Treatment[a]	Chlorophyll	Root oxidiz.	Chlorophyll	Root oxidiz.
CK	1.20 bB[b]	0.50 cB	1.44 cB	0.65 cB
Unic	1.43 aA	0.66 aA	1.71 aA	0.87 aA
WL	1.04 cC	0.38 dC	1.20 dC	0.51 dC
Unic + WL	1.39 aA	0.58 bB	1.57 bAB	0.73 bB

[a] Treatment description is given in Table 1.
[b] Within columns, means followed by the same small or capital letters are not significantly different at the 0.05 and 0.01 levels of probability, respectively.

Table 6. Effects of uniconazole and waterlogging on leaf and stem soluble sugar content (FW %) and seed yield (g/plant) of rape plants.

	Seedling stage		Flowering stage		
Treatment[a]	Leaf sugar	Stem sugar	Leaf sugar	Stem sugar	Seed yield
CK	7.68 bB[b]	16.20 bB	1.25 cB	6.70 aA	18.70 cC
Unic	8.13 bB	17.07 bB	1.33 cB	4.76 bB	29.35 aA
WL	13.95 aA	23.20 aA	2.51 aA	4.88 bB	13.80 dD
Unic + WL	13.65 aA	22.15 aA	2.34 bA	4.65 bB	22.29 bB

[a] Treatment description is given in Table 1.

[b] Within columns, means followed by the same small or capital letters are not significantly different at the 0.05 and 0.01 levels of probability, respectively.

Discussion

Unless efficiently metabolized, active oxygen species may alter plant metabolism by structurally modifying proteins and enhancing their susceptibility to proteolytic degradation (Pell and Dann 1991). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, whereas CAT and PODs metabolize H_2O_2 (Foyer et al. 1994). Our results indicated that waterlogging could weaken the enzymatic antioxidation systems of rape plants, thereby exposing them to oxidative stress (Table 1). Pretreatment of seedlings with uniconazole significantly increased SOD, CAT, and POD enzyme activities after waterlogging stress, which remained higher than the control and waterlogged treatment at the seedling and flowering stages. Our data are consistent with the hypothesis of Fletcher and Hofstra (1988) that triazole-induced stress tolerance in plants may be caused, at least in part, by increased antioxidant activity, which in turn reduces oxidative injury to membrane and/or enzyme activity.

Because the biosynthesis of unsaturated fatty acids requires O_2, the structure and function of cell membranes are expected to be sensitive to O_2 supply. Waterlogging significantly reduced the contents of unsaturated fatty acids detected in rape leaves at both the seedling and flowering stages of rape development (Tables 2 and 3). Oxidation of polyunsaturated fatty acids initiates lipid peroxidation chain reactions, generating additional free radicals (Mustafa 1990). In mitochondria extracted from yeast cells, degeneration under anoxia was closely associated with interference in the synthesis of unsaturated fatty acids, leading to the increased permeability of the inner mitochondrial membrane and uncoupling of ADP phosphorylation from respiration (Quinn and Champman 1980). Uniconazole plus waterlogging-treated plants had a significantly higher content of unsaturated fatty acids over the waterlogged plants at the seedling and flowering stages. These data suggest that uniconazole could increase waterlogging tolerance in relation to the desaturation of fatty acids of rape leaves.

Exposure of rape seedlings to 3 weeks of waterlogging significantly reduced the endogenous free proline content of leaves at both the seedling and flowering stages (Table 4). Proline accumulation has been considered as a symptom of stress-induced damage to plant tissue (Upadhyaya et al. 1989). Furthermore, it has been reported that treatments that increase proline accumulation, such as chilling, increase the tolerance of plants to stress (Flores et al. 1988). The prevention of oxidation of unsaturated vegetable oils by proline has also been reported (Ahmad et al. 1983). Pretreatment of rape seedlings with uniconazole significantly increased the endogenous free proline content of rape leaves in plants exposed to waterlogging, suggesting the involvement of proline in the protection of rape plants from waterlogging damage. MDA is a decomposition product from the peroxidation of polyunsaturated fatty acids (Chevrier et al. 1988). Increased electrolyte leakage is generally considered an index of membrane damage and deterioration (Simon 1974). There was a parallel increase in the level of lipid peroxidation (as indicated by MDA accumulation) and rate of electrolyte leakage from the leaves of rape plants exposed to waterlogging at both the seedling and flowering stages (Table 4). Uniconazole plus waterlogging-treated plants had a lower level of lipid peroxidation and rate of electrolyte leakage from the leaves at the seedling and flowering stages compared with the waterlogged plants. This may be due to the cumulative effects of uniconazole-induced enhancement of the antioxidation defense mechanisms of plants and the desaturation of fatty acids of the leaves.

The uniconazole plus waterlogging-treated plants were darker in color compared with the waterlogged plants and the control. Pretreatment of seedlings with uniconazole effectively delayed waterlogging-induced degradation of chlorophyll and reduction of root oxidizability (Table 5). Improved root oxidizability and root growth may have enhanced water and nutrient absorption. Dong et al. (1983) associated plant senescence with decreased root oxidizability and chlorophyll degradation.

There was a general increase in leaf- and stem-soluble sugar content after exposure of rape seedlings to waterlogging stress (Table 6). Pretreatment of rape seedlings with uniconazole did not alter the soluble sugar content of leaves and stems, after exposure to waterlogging stress. Pretreatment of seedlings with uniconazole increased the general performance of seedlings exposed to 3 weeks of waterlogging by producing higher seed yields. Uniconazole-induced alleviation of waterlogging damage in winter rape in relation to changes in morphological characteristics, hormones and photosynthesis had been reported in a separate paper (Leul and Zhou 1998).

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