IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

Characterization of pollen tube growth and induced molecular changes in *Berasicca napus* L. by UV-B treatment

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ABSTRACT INFO

Research Paper

Received: 11 Mar 2021

Accepted: 23 Nov 2021

ABSTRACT

During the past few decades, the problems caused by the stratospheric ozone reduction has stimulated remarkable research on higher plant responses to UV-B radiation. Depletion of the stratospheric ozone layer is leading to an increase in ultraviolet-B (UV-B: 280–320 nm) radiation reaching the earth's surface. This has raised interest in the possible consequences of increased UV-B levels on plant growth and development and the mechanisms underlying these responses. Although the effects of UV-B are now well characterized at the physiological level, little is known about the involved cellular and molecular mechanisms. Recent studies have shown that UV-B affects a number of important physiological processes, such as photosynthesis, through effects on gene expression. However, little research has addressed the reproductive biology of plants. The purpose of this study was to investigate the effects of UV-B radiation on reactive oxygen species (ROS) accumulation and antioxidant defense system in relation to germination and pollen tube growth of canola (*Brassica napus* L.). Our results illustrated that increased UV-B radiation decreased the pollen germination rate and tube length *in vitro*. Production of superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) increased by UV-B radiation treatment, and their accumulation resulted in lipid peroxidation. The activities and gene expression of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were decreased by enhanced UV-B radiation. The increased ROS and lipid peroxidation, as well as decreased antioxidant activities may be attributed to the effects of UV-B radiation on pollen germination and tube growth.

Key words: *Brassica napus* L., Pollen germination, Reactive oxygen species, UV-B radiation.

How to cite this article:
Navabpour S., Ebadi Almas D., and Pakdel M. (2021). Characterization of pollen tube growth and induced molecular changes in *Berasicca napus* L. by UV-B treatment. *Iranian Journal of Genetics and Plant Breeding,* 10(1): 1-9.
DOI: 10.30479/IJGPB.2021.15126.1290

ABBREVIATIONS

ANOVA (Analysis of variance), CAT (Catalase), H_2O_2 (Hydrogen peroxide), MDA (Malondialdehyde), $O_2^{\bullet-}$ (Superoxide anion radical), POD (Peroxidas), ROS (Reactive oxygen species), SOD (Superoxide dismutase).

INTRODUCTION

There is now little doubt that depletion of ozone in the earth's stratosphere is occurring and that the amount of ultraviolet- B (UV-B) radiation reaching the surface of the earth is thus increasing (Kerr and McElroy, 1993; Bais *et al*., 2019). The stratospheric ozone layer is the key factor in reducing solar UV-B radiation reaching the earth's surface. The measurable attenuation of the stratospheric ozone layer and the consequent increase in the terrestrial UV-B radiation showed a 6–14% increase since 1970s (UNEP, 2002; Bornman *et al*., 2019) which has raised interest in understanding the injurious effects of UV-B radiation on higher plants (Kataria *et al*., 2014). In addition, stratospheric ozone recovery may possibly be delayed due to a number of uncertainties, including interaction with other projected changes in global climate such as global warming (UNEP, 2008; Williamson *et al*., 2019). Therefore, it still remains interesting to investigate the effects of elevated UV-B radiation on different aspects of plant growth, continuously (Wang *et al*., 2006). UV-B can potentially affect seed quality in one of two ways. First, UV-B is a mutagen that may damage the embryo genetically, by causing mutations in the embryo itself or in the pollen and ovule that form the embryo (UNEP, 2008). Second, UV-B may stress the maternal plant (e.g., through disruption of photosynthesis; see reviews cited above) and affect the quantity or quality of resources provided to the seed. The effects of UV-B on seed quality have received little empirical study (UNEP, 2008; Williamson *et al*., 2019).

Important indices of male gametophyte functional ability are pollen germination capacity and the rate of pollen tube growth. Pollen of open flowers appear to be well shielded from solar UV-B when still within the anther sacs, but they may be exposed to natural UV-B radiation following dehiscence until successful germination and stigma penetration occur. Moreover, pollen grain walls can transmit as much as 20% of the UV-B (Stadler and Uber, 1942; Liu and Wang, 2021). Therefore, most of the studies concerning UV-B radiation on reproductive biology have focused on pollen. Previous results indicated that the reproductive function was influenced by increased UV-B radiation via pollination (He *et al*., 2006; Wang *et al*., 2010). Some studies, where pollen grains collected from healthy plants was directly exposed to UV-B by exposing the germination media to UV-B, showed that UV-B radiation reduced pollen germination (Feng *et al*., 2000; Benca *et al*., 2018). Torabinejad *et al*. (1998) investigated pollen grains of 34 taxa exposed to two levels of UV-B radiation. The pollen germination was inhibited in five tested species and pollen tube length was reduced in more than 50% of these species under high levels of UV-B radiation. In another study reported, growth and flowering period of *B. rapa* and *B. nigra* were decreased by increased UV-B exposure. Fitness, however, (estimated as seed number and seed quality) was generally elevated at the higher UV-B doses examined in our experiments. Offspring of UV-B exposed plants did not appear to suffer any major detrimental effects. Finally, neither floral traits nor attractiveness to pollinators was altered by the increased levels of UV-B (UNEP, 2008).

Living cells produce reactive oxygen species (ROS) under stressed and unstressed conditions creating oxidative stress leading to oxidative damage to lipids, proteins, nucleic acid etc. (Halliwell and Gutteridge, 1989; Hasanuzzaman *et al*., 2020). It was shown that the induction of ROS production was an early effect of UV-B radiation in plants (Agarwal, 2007; Reyes *et al*., 2019). He *et al.* (2006) and Wang *et al.* (2010) reported that ROS was involved in the UV-B-inhibited pollen germination and tube growth. To counter the hazardous effects of ROS, plants contain antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), as well as a wide array of nonenzymatic antioxidants (Kumari *et al*., 2010). The present paper reports effects of UV-B radiation on ROS accumulation and antioxidant defense system in relation to germination and tube growth of canola (*Brassica napus* L.) pollen.

MATERIALS AND METHODS

Pollen collection and irradiation

Canola (*B. napus* cv. Faclon) pollen was collected from flowers of approximately the same age. Approximately 0.1 g pollen was collected from ~1,000 flowers (30 plants) for each biological replicate, and three biological replicates were used for each analysis. Immediately after collection, pollen grains were transferred into petri dishes. The petri dishes were grouped into two, control (without UV-B radiation) and UV-B radiation treatment in the growth chamber (operated at 28 °C, 400 μmol m^{-2} s^{-1} photosynthetically active radiation

and 50–60% relative humidity). UV-B radiation was provided by four lamp tubes (Philips Tl 12/40w). The light was filtered with 0.13 mm thick cellulose diacetate (transmission down to 290 nm) for supplemental UV-B radiation or 0.13 nm thick polyester plastic film (absorbed all radiations below 320 nm) as control. The petri dishes were placed in the side of UV-B lamps and the desired UV-B radiation (0.5 W m^{-2}) was obtained by changing the distance between the lamps and the petri dishes. In order to get uniform exposure for all the pollen, the petri dishes were gently shaken every 5 min during the treatment process. In this study, *Brassica napus* pollen grains were exposed to UV-B radiation treatment for 0, 1, 2, 3 and 4 h. After the exposure, the pollen grains in each petri dish were separated into two portions. One portion was inoculated in the medium for measurement of pollen germination and tube length. The other portion was stored in liquid nitrogen for enzyme analyses.

Pollen germination and tube length *in vitro*

Pollen grains were cultured in the Hodgkin's and Lyons medium containing 9% sucrose and 13% polyethylene glycol (PEG-4000) (Rao., 1992) and incubated in light and high humidity for 4 h at either 23 °C or 35 °C. Germinating pollen grains (those with pollen tubes greater than twice the length of the pollen grain) were counted and photographed using a super high quality microscopic photographs, taken by a DP70 digital camera (Olympus Optical Co., Tokyo, Japan) interfaced to a BX51 Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Pollen germination was determined as a percentage of total pollen. Pollen tube length was acquired by measuring 20 randomly selected pollen from each replication using a microscope.

Measurement of superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) accumulation

Levels of $O_2^{\bullet-}$ were measured as described by Wang *et al*. (2006). The pollen grains (0.1 g) were homogenized in 1 mL 0.065 mol $L^{-}1$ phosphate buffer (pH 7.8), and the homogenate was centrifuged at 1000 *g* for 10 min. Then, 0.9 mL of the supernatant was collected and 50 μL 0.01 mol $L^{-}1$ hydroxylamine hydrochloride was added. After 20 min reaction at 25 °C, a mixture of 1 mL 1% α-naphthylamide and 1 mL 0.33% *p*-amino-phenylsulfonic acid were added. After 15 min reaction, 3 mL *n*-butanol was added into the reaction mixture which was then centrifuged at 6000 *g* for 10 min, and the specific absorption was read at 530 nm. Sodium nitrite was used as standard solution to make the standard curve for calculating the contents of $O_2^{\bullet-}$.

The contents of H_2O_2 in the pollen were quantified using the method of Patterson *et al*. (1984). The pollen grains (0.1 g) were homogenized in 3 mL cold acetone and the homogenate was centrifuged at 1500 *g* for 15 min at 4 °C; the resulting supernatant was collected and added to a concentrated hydrochloric acid solution containing 0.1 mL 20% $TiCl_4$ and 0.2 mL concentrated ammonia. After 10 min reaction at 25 °C, the reaction mixture was re-centrifuged at 1500 *g* for 20 min at 4 °C and then the pellets were washed twice with cold acetone and added into 3 mL H_2SO_4 (1 mM). The absorption was read at 410 nm and the content of H_2O_2 was quantified based on the standard curve.

Lipid peroxidation

Lipid peroxidation was determined through measurement of the malondialdehyde (MDA) contents. The pollen grains (0.1 g) were homogenized in 5 mL 50 mM potassium phosphate buffer (pH 7.0, containing 1% Polyvinylpyrrolidone (PVP); PVP and 1 mM ethylenediamine tetraacetic acid; EDTA). The homogenate was centrifuged (15000 *g* at 4 °C) for 20 min and the supernatant was used for MDA assay. The MDA content was measured according to Heath and Packer (1968).

Antioxidant enzyme assays

The pollen grains (0.1 g) were homogenized in 3 mL of 50 mM potassium phosphate buffer (pH 7.0, containing 1% Polyvinylpyrrolidone (PVP)). The homogenate was centrifuged (15000 *g* at 4 °C) for 15 min and the supernatant was used as an enzyme extract to determine SOD, CAT and POD activities. Protein concentration of the enzyme extract was determined according to Bradford (1976), based on a standard curve pre-established with bovine serum albumin. SOD (EC 1.15.1.1) activity was determined as described by Beauchamp and Fridovich (1971). CAT (EC 1.11.1.6) activity was measured spectrophotometrically according to Chance *et al*. (1979). POD (EC 1.11.1.7) activity was assayed according to Tewari *et al*. (2005).

Gene expression analysis

To evaluate the expression of genes encoding superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), total RNA was extracted from 100 mg of samples prepared using the Biozol kit (Bioflex-Japan). These genes encode the enzymes involved in hemostatic cell buffering by mopping up the intensive amount of reactive oxygen species. After DNaseI treatment, extracted RNA was quantified using a spectrophotometer (Uvikon, Kontron, Zurich, Switzerland) and its quality was verified using 1.5% agarose gel electrophoresis. The cDNA strand was

Table 1. Analysis of variance for studied traits.

Source of variation	df	Mean of square							
		PGR	PTL	MDA	$O_2^{\bullet-}$	H_2O_2	SOD	CAT	POD
UVB treatment	1	351**	451**	0.21**	35.9*	135**	73.2**	131**	182**
Time	4	566**	891**	0.45**	96.1**	275**	96.2**	245**	231**
UVB×Time	4	278**	221*	0.11**	55.9**	35.1^{ns}	19.2^{ns}	45.1^{ns}	33.1^{ns}
Error	18	56.2	131	0.05	22.7	33.5	22.3	40.2	30.2
Coefficient of variation (%)	-	4.2	6.5	3.1	2.8	4.1	3.5	1.6	3.3

PGR: Pollen germination rate, PTL: Pollen tube length, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase.
^{ns}: not significant difference (P>0.05) and the significances are indicated: *P<0.05, **P<0.01.

synthesized from 1 µg total RNA as the template with an RT reagent kit (Fermentas company, Vilnius, Lithuania), in accordance with the manufacturer's protocol. The specific primers for quantitative real-time (qRTPCR) PCR were designed using Primer3 software. The housekeeping gene GAPDH was used as endogenous control (Gonc¸alves *et al.,* 2005). The qRT-PCR was implemented in an iCycler iQ5 thermocycler (Bio Rad Company). The quantity of mRNAs was measured by SYBR Green method using SYBR Biopars Kit (GUASNR, Iran). Each sample was evaluated in 3 repetitions and two biological replications. Relative gene expression was calculated by Pfaffl *et al*. (2001) formula, $2^{-\Delta\Delta CT}$ in relation to control samples (0, non-use).

Statistical analysis

The germination data were tested for normality before analysis of variance. Data were analyzed using SAS software (Version 9.2, SAS Institute Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) was performed on all results and differences between the means were compared using Duncan values (P<0.05). To evaluate the relationship among variables, they were also subjected to Pearson correlation analysis based on data matrix. Analysis of the data obtained from gene expression was performed using REST software (Pfaffl *et al.,* 2002).

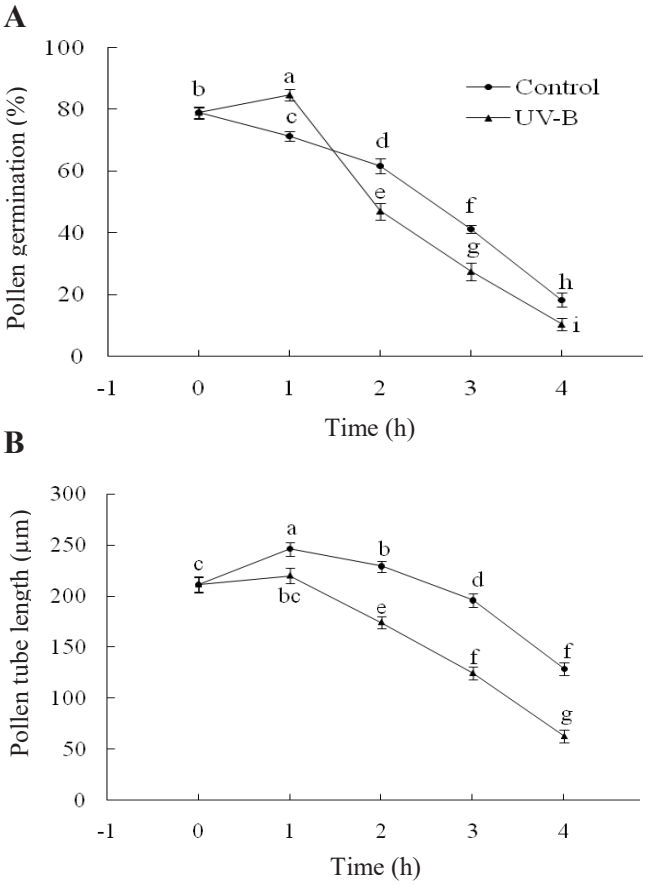

Figure 1. Effects of UV-B radiation on **A:** pollen germination, and **B:** pollen tube length of *Brassica napus*. Data are the means of three replicates±SE. Means with different letters are significantly different at P<0.05.

RESULTS

The results of variance analysis showed UVB treatment and time course affected all treatments significantly. Also, there were significant interaction effects for PGR, PTL, MDA and $O_2^{\bullet-}$ that indicated the effect of UVB treatments were not independent of time course (Table 1). The small values of C.V indicated accuracy of experiments.

Both the pollen germination rate and tube length decreased as time of exposure increased, in all UV-B radiation treatments (Figure 1A). The pollen germination rate decreased continuously for up to 4 h under control condition. Under UV-B radiation treatment, the germination rate increased from 79% to 84.6% in the first 1 h treatment, and then decreased significantly to 47% after 2 h, 27.5% after 3 h and

Table 2. Correlation among pollen germination rate, pollen tube length, reactive oxygen species accumulation, lipid peroxidation and antioxidant enzyme activities.

Trait	PGR	PTL	MDA	$O_2^{\bullet-}$	H_2O_2	SOD	CAT
PTL	0.97**						
MDA	-0.86**	-0.81**					
$O_2^{\bullet-}$	-0.90**	-0.85**	0.96**				
H_2O_2	-0.81**	-0.77**	0.98**	0.91**			
SOD	0.77**	0.72**	-0.87**	-0.75**	-0.90**		
CAT	0.87**	0.82**	-0.87**	-0.79**	-0.88**	0.96**	
POD	0.73**	0.86**	-0.48^{ns}	-0.57*	-0.45^{ns}	0.38^{ns}	0.49^{ns}

PGR: Pollen germination rate, PTL: Pollen tube length, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, POD: Peroxidase.
^{ns}: not significant difference (P>0.05) and the significances are indicated: *P<0.05, **P<0.01.

10.4% after 4 h (Figure 1A). The results indicate that the pollen tube length was sensitive to the elevated UV-B treatment (Figure 1B). Under control conditions, the pollen tube length was only decreased by 39% after exposure for 4 h, while under UV-B radiation treatment, it was decreased by 41% and 70% after exposure for 3 and 4 h, respectively (Figure 1B).

The changes in $O_2^{\bullet-}$ and H_2O_2 contents are shown in Figure 2. Both $O_2^{\bullet-}$ and H_2O_2 production increased after the pollen grains were exposed to the UV-B radiation. UV-B radiation enhanced more $O_2^{\bullet-}$ production when the pollen grains were exposed to the elevated UV-B for 3 and 4 h (Figure 2A). The contents of H_2O_2 in the pollen increased progressively with the time of exposure for both control and the UV-B radiation treatment. However, H_2O_2 content was found to be higher in UV-B radiation than control (Figure 2B). Under control conditions, H_2O_2 production ranged from 23 to 45 μmol g^{-1} after 4 h. While under UV-B radiation treatments, H_2O_2 production ranged from 23 to 69 μmol g^{-1}. Also, H_2O_2 production showed a negative correlation with pollen germination rate (r=-0.81, P<0.01) and pollen tube length (r=-0.77, P<0.01) (Table 2).

MDA contents increased with time of exposure for both control and the UV-B radiation treatments (Figure 3). However, UV-B radiation enhanced more MDA accumulation rapidly. Under UV-B radiation, the MDA content increased significantly after exposure for 1 h, but it significantly increased after 2 h under control conditions (Figure 3). There was a strong direct correlation between MDA and H_2O_2 (r=0.98, P<0.01), as well as between MDA and $O_2^{\bullet-}$ (r=0.96, P<0.01) (Table 2). In addition, the MDA had negative correlations with pollen germination rate (r=-0.86, P<0.01) and pollen tube length (r=-0.81, P<0.01) (Table 2).

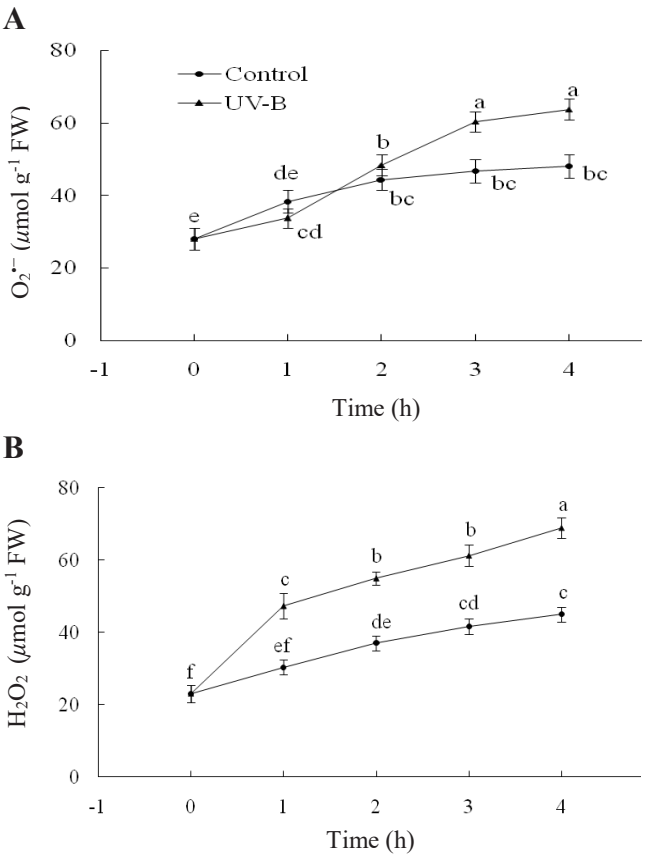

Figure 2. Effects of UV-B radiation on **A:** $O_2^{\bullet-}$, and **B:** H_2O_2 accumulation in *Brassica napus* pollen. Data are the means of three replicates±SE. Means with different letters are significantly different at P<0.05.

The activities and gene expression of SOD and CAT showed a decreasing trend with the time of exposure, however, it was higher under UV-B treatment than control (Figures 4A, 4B and 5), suggesting that increased UV-B radiation inhibited their activities and gene expression. However, the activities and gene expression of POD were first increased up to 2 h of exposure, and

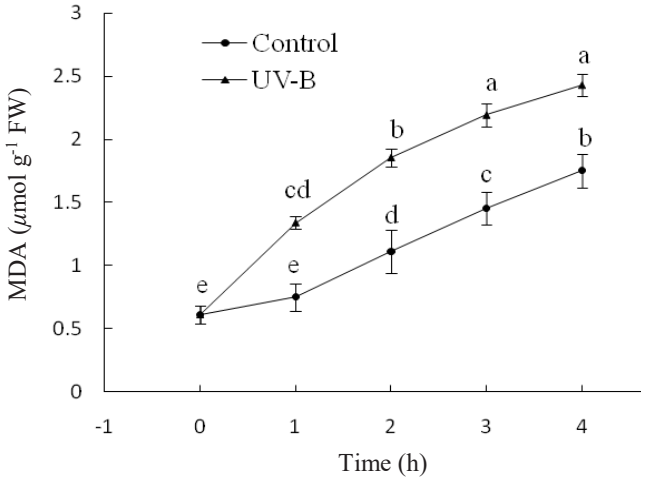

Figure 3. Effects of UV-B radiation on lipid peroxidation (malondialdehyde [MDA] content) in *Brassica napus* pollen. Data are the means of three replicates±SE. Means with different letters are significantly different at $P<0.05$.

then decreased, rapidly. UV-B radiation also inhibited POD activities (Figure 4C). The activities of SOD and CAT showed a negative correlation with $O_2^{\bullet-}$ contents (r=-0.75, $P<0.01$) and H_2O_2 contents (r=-0.88, $P<0.01$), respectively (Table 2).

DISCUSSION

Role of oxidative stress due to UV-B radiation in the form of pollen germination, pollen tube growth, ROS accumulation and antioxidant enzyme activities were studied. Results of our investigation confirmed previous reports that UV-B radiation decreased pollen germination and tube length (He *et al*., 2006; Torabinejad *et al*., 1998). In plants, several metabolic processes produce ROS, including superoxide radicals, H_2O_2, hydroxyl radicals and other free radicals. The production of ROS was substantially induced by environmental stresses (Mittler, 2002). Wang *et al*. (2010) showed that UV-B radiation induced ROS production during pollen germination. In our research, the elevated UV-B radiation increased both $O_2^{\bullet-}$ and H_2O_2 accumulation, which increased progressively with the time of UV-B radiation treatment (Figure 2). It is well known that the over production of ROS in living organisms under stress conditions is potentially toxic to the normal metabolism and results in oxidative damage, such as lipid peroxidation, protein degradation and DNA damage (De Jager *et al*., 2017; Hasanuzzaman *et al*., 2019].

Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids hydroperoxides, has been

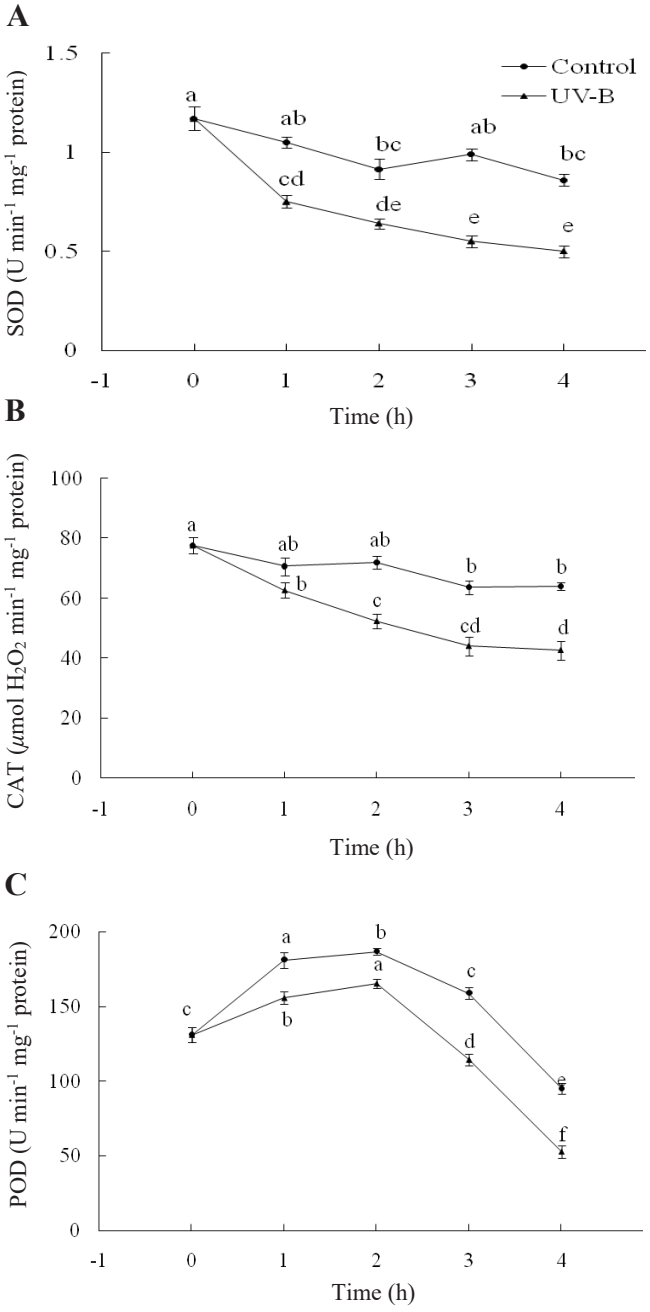

Figure 4. Effects of UV-B radiation on antioxidant enzyme activities in *Brassica napus* pollen. **A:** superoxide dismutase (SOD), **B:** catalase (CAT) and **C:** peroxidase (POD). Data are the means of three replicates±SE. Means with different letters are significantly different at $P<0.05$.

employed very often as a suitable biomarker for lipid peroxidation (Ayala *et al*., 2014), which is an effect of oxidative stress. Our results show that MDA content in pollen grains was increased by exposure to UV-B radiation (Figure 3). Shivanna and Sawhney (1997) reported that pollen germination was closely associated with the stability of the pollen cell membrane. Our results indicated a negative correlation between pollen

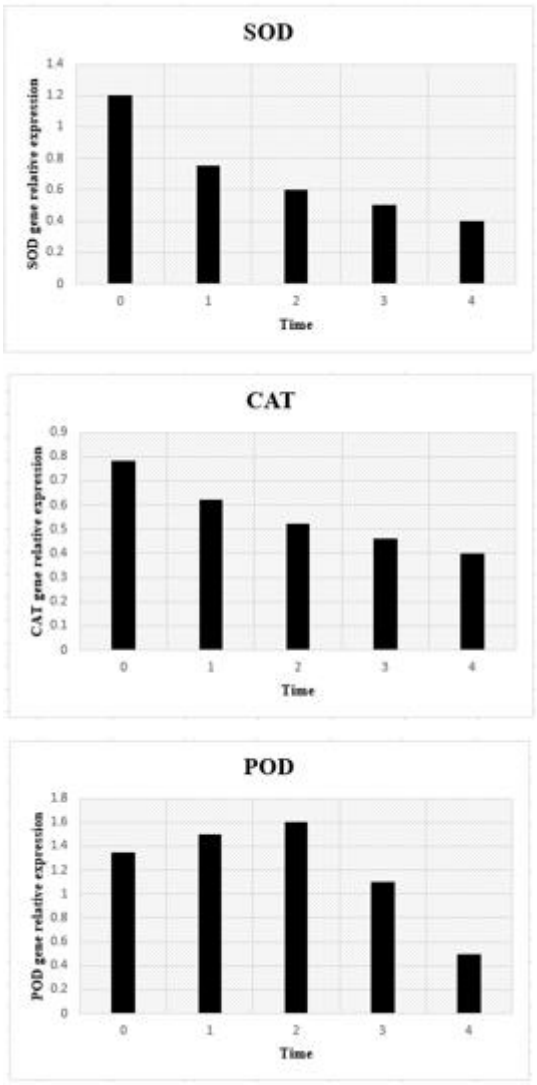

Figure 5. Effects of UV-B radiation on superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) gene expression.

germination rate and MDA concentration (r=-0.86, P<0.01). Accordingly, the lipid peroxidation induced by UV-B radiation was responsible for the loss of pollen germination.

ROS scavenging is a common response to most stresses and depends on the detoxification mechanism provided by an integrated system of nonenzymatic and enzymatic antioxidants (Ahmad *et al*., 2008). SOD, CAT and POD are the key enzymes of the antioxidant defense system. SOD accelerates the conversion of superoxide to H_2O_2, while CAT and POD catalyze H_2O_2 breakdown. In this study, the activities of SOD, CAT and POD in canola pollen were decreased by the elevated UV-B radiation. This is in agreement with those findings reported earlier on maize (*Zea mays* L.) pollen by Wang *et al*. (2010). The decreased activities of SOD, CAT and POD indicate that UV-B radiation impaired the antioxidant activity of the pollen. This may contribute to the highly negative correlations between SOD and ($O_2^{\bullet-}$) (r=-0.75, P<0.01), and between H_2O_2 and CAT (r=-0.88, P<0.01) (Table 2). Similar results were also reported in other plants (Wang *et al.,* 2010; Agrawal and Rathore, 2007).

Recent studies have provided that the pollen germination and tube growth are mediated by Ca^{2+} (Wu *et al*., 2011; Wu *et al*., 2011). Flux of Ca^{2+} through the Ca^{2+}-permeable channels into a particular region of the grain where the pollen tube will emerge, or into pollen tube tip, maintains a steep tip-to-base cytoplasmic Ca^{2+} gradient, which controls pollen germination or pollen tube growth through regulation of membrane vesicle fusion and cytoskeletal dynamics (Holdaway-Clarke *et al*., 1997), and hence abolishing the tip-to-base cytoplasmic Ca^{2+} gradient by influencing Ca^{2+} flux inhibits pollen germination and tube growth (Obermeyer and Weisenseel, 1991). Besides, some studies have also shown that H_2O_2 regulates stomatal movement through the activation of Ca^{2+} channels (He *et al*., 2006). Thus, it is very interesting to know whether H_2O_2 can influence pollen germination and tube growth by mediating the Ca^{2+} channel. In general, our results showed that expression and activity of antioxidant enzymes (SOD, CAT and POD) increased following plant treatment with UV-B radiation.

ACKNOWLEDGEMENTS

The authors are grateful to Agricultural Sciences & Natural Resources University for financial support.

Ethics approval

The authors completed all ethical aspects.

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