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RESEARCH ARTICLE

PHYSIOLOGICAL AND ANTHOCYANIN BIOSYNTHESIS GENES RESPONSE INDUCED BY VANADIUM STRESS IN MUSTARD GENOTYPES WITH DISTINCT PHOTOSYNTHETIC ACTIVITY

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Highlights:

- Vanadium affected the photosynthetic performance of mustard genotypes.
- Antioxidant enzyme activities were also increased under vanadium stress.
- The anthocyanin and soluble protein contents were decreased with increasing vanadium.
- TT8, F3H and MYBL2 genes were associated with anthocyanin synthesis in purple mustard.
- The purple exhibited higher tolerance to vanadium stress than the green genotype.

Abstract

The present study aimed to elucidate the photosynthetic performance, antioxidant enzyme activities, anthocyanin contents, anthocyanin biosynthetic gene expression, and vanadium uptake in mustard genotypes (purple and green) that differ in photosynthetic capacity under vanadium stress. The results indicated that vanadium significantly reduced photosynthetic activity in both genotypes. The activities of the antioxidant enzymes were increased significantly in response to vanadium in both genotypes, although the purple exhibited higher. The anthocyanin contents were also reduced under vanadium stress. The anthocyanin biosynthetic genes were highly expressed in the purple genotype, notably the genes TT8, F3H, and MYBL2 under vanadium stress. The results indicate that induction of TT8, F3H, and MYBL2 genes was associated with upregulation of the biosynthetic genes required for higher anthocyanin biosynthesis in purple compared with the green mustard. The roots accumulated higher vanadium than shoots in both mustard genotypes. The results indicate that the purple mustard had higher vanadium tolerance.

Keywords:

Vanadium; *Brassica;* Anthocyanins; Gene expressions; Photosynthetic activity

1 Introduction

Heavy metal pollution of agricultural soils is one of the major environmental concerns, adversely affecting the yield and the quality of crops. Vanadium (V) is the 5th most abundant transition metal and is widely distributed (~0.01%) in the earth' crust (Amorim et al., 2007). Vanadium naturally exists in several mineral forms such as carnotite, patronite, chileite and vanadinite, with varied distribution ranges (3-310 mg kg^{-1}) in soils. Recently, accumulated evidence of the increasingly higher vanadium level in the atmosphere has raised concerns over its potential ecological hazard due to vanadium associated anthropogenic activities (Teng et al., 2011). Vanadium is considered as an active component of the vanadium biogeochemical cycle in the surface environment, due to its high mobility. Recent ecotoxicological reports have placed vanadium among the dangerous pollutants, in the same class as mercury and lead (Naeem et al., 2007; Baken et al., 2012).

The significance of vanadium for various biological functions, such as nitrogen-fixing bacteria and various soil fungi, has been identified very recently (Anke, 2004). Moreover, vanadium-associated toxicity affects living organisms, including plants (Qian et al., 2014; Tian et al., 2014a). Vanadium toxicity can seriously impair root and leaf morphology that may lead to complete growth inhibition and even plant death (Saco et al., 2012; Imtiaz et al., 2015). A recent study demonstrated that vanadium pollution of industrial red mud was responsible for noticeable chromosomal damages in pollen assembly and root cells of food crops (Misik et al., 2014).

Heavy metals induce formation of reactive oxidation species (ROS), superoxide anion (O^{2-}), hydroxyl (OH^{-}) radicals, and H_2O_2 that in turn damage the chloroplast and the photosynthetic efficiency. The presence of H_2O_2 in the chloroplasts restricts the Calvin-cycle enzymes and reduces carbon assimilation (Takeda et al., 1995). These changes disrupt key cell membrane functions, such as the regulation of the peroxidation process of polyunsaturated fatty acids (De Vos et al., 1993) and the reduction in the oxidative damage caused by the formation of oxygen free radicals and/or by the reduction in the status of enzymatic and non-enzymatic antioxidants

(Somashekaraiah et al., 1992; Shaw, 1995). Various plants exhibit a wide array of defense mechanisms in order to protect chloroplast and cellular membranes from ROS (Foyer and Harbinson, 1994). The plants have evolved an antioxidant defense system against stressors, including heavy metals. Superoxide dismutase (SOD) activates primary cellular defense response in the stressed plant. It catalyzes partitioning of superoxide radicals (O^{2-}) into H_2O_2 and O_2, whilst the accumulation of H_2O_2 in the cell membrane is restricted by catalase (CAT) (Noctor et al., 2002).

Anthocyanins are a flavonoid subclass abundantly present in several plant tissues. These water-soluble pigments are initially produced in the cytoplasm and are later transported into the vacuole (Jaakola et al., 2002; Gould, 2004). Anthocyanins are produced by plants in response to various stresses. They are often associated with enhanced stress tolerance in the plant and upregulation of the antioxidant defense system under stressful conditions (Neill et al., 2002; Merzlyak et al., 2008).

The anthocyanin biosynthesis pathway is one of the key components of flavonoid metabolism, and has been extensively studied in several plant species. The biosynthesis of anthocyanins is initiated by sequential enzyme reactions involving chalcone synthase (CHS), chaclone isomerase (CHI) and flavanone 3-hydroxylase (F3H) (Holton and Cornish; 1995; Winkel-Shirley, 2001). However, the enzyme dihydroflavonol 4-reductase (DFR) catalyzes the NADPH-mediated reduction of dihydroflavonols to leucoanthocyanidins as an immediate precursor of anthocyanidins (Lo Piero et al., 2006). In view of their importance in the regulation of plant stress, certain studies have examined genes that are related to the anthocyanin biosynthesis. Notably, CHS and DFR genes have been isolated from several higher plants and are relatively well characterized. The induction of CHS and DFR gene expression as well as that of other genes involved in anthocyanin biosynthesis has been reported under stress conditions, including stress caused by metal exposure (Zhang et al., 2014). However, a paucity of studies are available

on the expression levels of genes involved in *Brassica* plants under heavy metal stress conditions, particularly vanadium exposure.

The ability of plants to accumulate heavy metals in their roots and aerial plant parts depends upon the type of metal and its concentration in the growth medium as well as the plant species (Sobukola and Dairo, 2007). The level of metal accumulation into different plant parts is fundamental in order to screen for their metal hypertolerance and/or exclusion (Sekhar et al., 2001). Mustard is a well-known model hyperaccumulator plant that effectively removes various metal contaminants. The diverse photosynthetic capacity of the mustard plant to detoxify ROS and to protect key chloroplast functions against metal induced oxidative stress highlights its wider applicability in metal contaminated soil. The mechanism of anthocyanin biosynthesis and composition, which is considered a key stress adaptive measure, in the mustard plant under vanadium stress is unknown.

To date, the dynamics of vanadium in plants are not fully known. The major objective of the present work was to study the physiological performance of genotypes that exhibited different levels of anthocyanins. Therefore, the objectives of the present study were to (1) to evaluate the influence of vanadium stress on anthocyanin accumulation in leaves of purple mustard plants, (2) to investigate the mechanism of the anthocyanin biosynthesis in the purple mustard, the induction of the expression of structural and regulatory genes in two genotypes, namely purple genotype (Ziyejiecai) and green genotype (G19). In addition, the current study aimed (3) to evaluate the effect of vanadium stress on the net photosynthetic rate (*P*n), stomatal conductance (*g*s), transpiration rate (*T*r) in mustard genotypes differing in photosynthetic capacity, and finally (4) to evaluate the expression levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in mustard genotypes with different photosynthetic activity.

2 Materials and methods

2.1 Plant material and growth conditions

Mustard (*Brassica juncea* var. gracilis) was used as a test plant in this experiment. Seeds of purple genotype (Ziyejiecai) and green genotype (G19) that differed in photosynthetic capacity were used. The seeds of selected mustard genotypes were surface sterilized and sown into the peat moss mixed plastic pots (170 mm-220 mm). At five leaf stage, seedlings of uniform size were selected and transferred into small plastic pots with plate holes. A total of 5 plants were maintained in each pot supplemented with half strength Hoagland nutrient solution. The pH of the nutrient solution was adjusted at 5.5 with NaOH and/or HCl. The hydroponic system was provided with continuous air flow using a pump equipped with a pre-filtered membrane. The hydroponic nutrient solution was renewed once a week. The plants were kept in a growth chamber at 22 ± 2°C and relative humidity of 60-70%. A photoperiod of 14 h light with an average photon flux density of 820 µmol m^{-2} s^{-1} was maintained by an assembly of cool–white fluorescent lamps. The experimental design was conducted following completely randomized design (CRD) with three replicates.

Following an acclimatization period of 7 days, vanadium as ammonium metavanadate (NH_4VO_3) was added into full strength Hoagland nutrient solution according to the following 5 treatments: (1) control (without vanadium), (2) vanadium @ 20 mg L^{-1}, (3) vanadium @ 40 mg L^{-1}, (4) vanadium @ 80 mg L^{-1}, (5) vanadium @ 100 mg L^{-1}. Following 60 days, the plants were harvested for plant biochemical and molecular analysis, while prior to harvesting, photosynthetic parameters were recorded. The plants were washed and rinsed with Milli-Q water prior to separation into leaves, shoots, and roots. Fully expanded leaves were harvested, and placed in liquid nitrogen, and stored at −80°C for further analysis. For the determination of vanadium, the plants were dried at 100°C for 24 h, and then at 60°C till constant weight.

2.2 Photosynthetic activity measurement

The photosynthetic parameters [net photosynthetic rate (*P*n), stomatal conductance (*g*s) and

transpiration rate (*Tr*)] were measured using the portable photosynthesis system (LiCor-6400 LI-COR Inc., Lincoln, NE, USA). These measurements were recorded on the upper leaf of the main branch that was expanded to the maximum length. The growth conditions of the cabinet at the time of measurement were as follows: photosynthetic active photon 1000 µmol m^{-2} s^{-1}, relative humidity 60±3%, temperature 20±2°C. A total of 3 readings per treatment were taken from randomly selected plants (Sing et al., 2007).

2.3 Antioxidant enzymes and soluble protein assay

Fresh leaves (0.5g) were finely crushed in liquid nitrogen, and resuspended in 5 ml of 0.2 mol L^{-1} sodium phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was collected and used as a source for antioxidant enzymatic assays. Superoxide dismutase (SOD) activity was determined by the method of Beauchamp and Fridovich (1971). Catalase (CAT) activity was assayed using the method illustrated by Aebi and Bermeyer (1983). Peroxidase (POD) activity was measured using the guaiacol oxidation method (Upadhyaya et al., 1985). The soluble protein contents were determined by the method of Bradford (1976).

2.4 Anthocyanin extraction and analysis

The total anthocyanin content in plant samples was analyzed using the pH-differential spectrum method (Rapisarda et al., 2000). The mature leaves (100 mg) were frozen in liquid nitrogen and finely crushed into powder. The leaves were resuspended separately in 2 mL of pH 1 buffer solution (50 mM KCl and 150 mM HCl) and 2 mL of pH 4.5 buffer solution (400 mM sodium acetate and 240 mM HCl). The mixtures were centrifuged at 12,000g for 20 min at 4°C. The absorbance of the solutions was measured at 520 nm using diluted supernatants. The anthocyanin content was calculated according to the following equation:

$$\textbf{Anthocyanin}\ (\textbf{mg g}^{-1}\ \textbf{FW}) = (\textbf{A1} - \textbf{A2}) \times {}^{449.2}/_{34300} \times \textbf{DF}$$

Where:

A1 = Absorbance of supernatants gathered from pH 1 buffer solution at 520 nm,

A2 = Absorbance of supernatants gathered from pH 4.5 buffer solution at 520 nm,

449.2 = Molecular mass of cyanidin-3-glucoside,

34300 = Molar absorptivity of cyanidin-3-glucoside at 520 nm and

DF = Dilution factor

2.5 RNA extraction and complementary DNA (cDNA) synthesis

Under hydroponic conditions, young leaves were collected at the same time after one month of treatment application. The samples were immediately frozen in liquid Nitrogen and stored at -80 °C until further analysis. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The quality of purified RNA was initially evaluated on an agarose gel and the samples were subsequently quantified using a Nano Drop spectrophotometer (Thermo Fisher Scientific, Inc.). The integrity of RNA samples was further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). A total of 1 to 2 μg of total RNA was used to synthesize the complementary DNA (cDNA) by the enzyme reverse transcriptase (M-MuLV RT) (Promega) in the presence of Oligo-dT18 primers. The synthesized cDNA samples were appropriately diluted with RNase/DNase-free water.

2.6 Gene expression analysis using Semi quantitative RT-PCR

Reverse transcription was performed by Super Script III Reverse Transcriptase (Invitrogen) and oligo (dT) according to the manufacturer's instructions. The sequences of the selected genes were obtained from the *Brassica oleracea* genome database. Forward and reverse primers were designed using the Primer 3 software. β-actin, was the internal housekeeping gene control. The specific gene primers (Table 1) were used to amplify target genes from different materials (32 cycles). The amplification reactions were conducted at the following conditions: an initial denaturation step at 94°C for 5 min, 32 cycles of dentaruration at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension step at 72°C for 10 min. The samples were subsequently stored at 4°C until further analysis. The samples were analyzed by gel electrophoresis in order to verify the specificity of the PCR.

2.7 Vanadium determination

Vanadium contents of treated roots and shoots of mustard genotypes were determined according to Kashif et al. (2009) using graphite furnace atomic absorption spectrophotometry (GFAAS-GTA 120).

2.8 Statistical analysis

The data were tested for normal distribution and homogeneity using multiple tools, and were always log-transformed when necessary prior to the statistical analysis. We performed multivariate analysis of variance (MANOVA) test using vanadium concentration and mustard genotype as fixed factors. The results were always presented as means ± SD of three independent replicates in the figures, unless otherwise stated. The statistical analyses were performed using SPSS for windows software v.21 (SPSS, Chicago, IL, USA).

3 Results

3.1 Plant growth

The changes in the shoot and root lengths of the plants and their appearance are considered highly sensitive plant growth response parameters under stress conditions. The treatment of the plants with various concentrations of vanadium (20-100 mg L^{-1}) significantly reduced growth and development of shoots and roots in both mustard genotypes (Fig. 1). However, the growth of the green genotype plants was more adversely affected by vanadium stress compared with the purple genotype plants. In addition, we observed that the increase in vanadium concentrations further inhibited the lateral root development in both genotypes.

3.2 Photosynthetic activity

The vanadium treatments resulted in significant ($p<0.001$) reductions in the photosynthetic parameters in both mustard genotypes (Fig. 2; Table 2). In the absence of vanadium, the stomatal conductance (*g*s) and transpiration rate (*T*r) were higher in the green mustard genotype compared with the purple mustard genotype, which also explained the higher rate of photosynthesis observed in the green mustard genotype (Fig. 2-B & C)**.** The stomatal conductance (*g*s) and

transpiration rate (*T*r) were significantly enhanced following treatment with vanadium concentrations of 20, 40 and 100 mg L^{-1} in the purple mustard genotype compared with that noted in the green mustard genotype. The rate of photosynthesis (*P*n) was lower in the purple mustard genotype compared with the green mustard genotype for the control and 80 mg L^{-1}, vanadium treatments respectively (Fig. 2-A). However, the rate of photosynthesis (*P*n) was higher in the purple mustard genotype following vanadium treatment of 40, 80, and 100 mg L^{-1} due to the enhanced stomatal conductance (*g*s) and transpiration rate (*T*r) compared with that noted in the green mustard genotype. The vanadium treatment caused significant difference with regard to the photosynthetic parameters of the mustard genotype plants *($p<0.01$).*

3.3 Response of antioxidant enzymes

The results indicated positive relationships among the vanadium concentrations and antioxidant enzyme activities in the mustard plants; however, the mustard genotypes differed significantly in response to the various vanadium treatments (Fig. 3; Table 2). The superoxide dismutase (SOD) activity in both mustard genotypes was significantly (*$p<0.001$*) higher in vanadium treated plants compared with that noted in the control treatment plants (Fig. 3-A). The activity of SOD was approximately 1.5, 2.3, 2.7, and 3.2 folds higher compared with the control samples for the green mustard genotype at 20, 40, 80, and 100 mg L^{-1} of vanadium treatment, respectively. However, the purple mustard genotype indicated higher SOD activity compared with the green mustard genotype, at 20, 40, 80, and 100 mg L^{-1} of vanadium treatment, respectively. The activity measured corresponded to an approximate 2.2, 2.6, 3.2, and 3.8 folds higher activity compared with the control treatment.

The activity of catalase (CAT) was significantly different (*$p<0.001$*) and consistently increased in both mustard genotypes following treatment with varying vanadium concentrations compared with the control samples (Fig. 3-B). The results indicated that the green mustard genotype exhibited approximately 4.3, 5.6, 7.1, and 10.7 folds higher CAT activity compared with the control treatment in plants that were treated with 20, 40, 80 and 100 mg L^{-1} of vanadium,

respectively. The purple mustard exhibited 5.2, 6.6, 8.3, and 12.6 folds higher CAT activity compared with the control samples following application of vanadium at 20, 40, 80, and 100 mg L^{-1}, respectively.

The addition of vanadium significantly ($p<0.001$) increased the peroxidase (POD) activity compared with the control samples in both mustard genotypes (Fig. 3-C). The green and purple mustard genotype indicated approximately 1.8, 3.0, 4.5 and 8.3 folds, and approximately 2.2, 4.3, 7.7, and 10.2 folds increased POD activity compared with the control samples, respectively following treatment with 20, 40, 80 and 100 mg L^{-1} of vanadium. The data demonstrated that the purple mustard genotype exhibited higher activity of SOD, CAT, and POD enzymes compared with the green mustard genotype.

3.4 Analysis of anthocyanin contents and expression profiles of biosynthesis genes

The accumulation of anthocyanin in the leaves of the purple mustard plants declined sharply with increasing concentrations of vanadium (Fig. 4). Only trace amounts of anthocyanin contents were observed in the leaves treated with the highest vanadium concentrations of 100 mg L^{-1} compared to the treatments that included lower vanadium concentrations as well as the control treatment. Moreover, the anthocyanin content of the green mustard leaves further decreased with the increase in vanadium concentrations, although it remained below 0.1 mg g^{-1} FW. In order to determine the relationship between the anthocyanin content and the expression of the anthocyanin biosynthetic genes, their corresponding transcripts were examined in the leaves of the purple and green genotypes. The expression levels of the phenylpropanoid early pathway and late biosynthetic genes, namely PAL2, PAL4, C4H, 4CL, CHS, CHI and F3H were investigated (Fig. 5). Following 30 days of vanadium treatment, the leaves of the purple and green genotypes were compared with both control and vanadium treated plants. The expression levels of the anthocyanin genes in the purple genotype were high in the leaves of the control treatment. The expression levels of these structural genes reached their peak in the purple mustard genotype leaves at the time point 0 of the growth under vanadium treatment. In the purple mustard

genotype leaves, the structural genes were markedly up-regulated. The expression levels of various genes were highly up-regulated in the leaves of purple genotype compared with the green mustard genotype. Despite the absence of visual evidence of anthocyanin pigmentation in the corresponding tissues of the green genotype, the low expression levels of these structural genes were observed (Fig. 5). The high expression levels of the biosynthetic genes were strongly associated with the high intensity of anthocyanin pigments in the purple mustard. The results indicated that the adequate levels of transcripts of some vital structural genes were essential for a large amount of anthocyanin biosynthesis. Moreover, the results were completely consistent with their phenotype and further illustrated that the expression levels of the structural genes were closely related to the anthocyanin accumulation in purple mustard.

At low vanadium concentrations (20 mg L^{-1}), the maximum expression levels of the genes PAL2, C4H, CHS, F3H and TT19 were observed in the purple mustard leaves. However, PAL4 and CHS indicated maximum induction in the purple mustard leaves at vanadium concentrations of 40 mg L^{-1}. The expression levels of these genes was reduced gradually. CHI indicated the maximum expression levels at 100 mg L^{-1} of vanadium treatment. In the present study, the genes TT8, F3H, and MYBL2 were expressed at all vanadium treatments in the purple mustard genotype. The data further indicated that the induction of TT8, F3H, and MYBL2 genes was essential in order to retain the production of the anthocyanin contents. Therefore, these genes correlated with the upregulation of the anthocyanin biosynthesis in the purple mustard compared with the green mustard following vanadium treatment at different concentrations. The semi qRT-PCR results revealed that the anthocyanin contents gradually decreased with the increase in vanadium stress in both genotypes, possibly due to toxic effects (Fig. 5).

3.5 Soluble protein contents

The vanadium treatment significantly ($p<0.001$) reduced the protein contents in both mustard genotypes (Fig. 6). The green mustard genotype exhibited a reduction in protein contents of 19.3%, 37%, 55% and 71% following vanadium treatment at 20, 40, 80 and 100 mg L^{-1},

respectively compared with the control treatment. In contrast to the green mustard, the purple mustard genotype followed similar reduction in protein contents following the aforementioned vanadium concentration treatments. However, the percentage decrease noted following vanadium treatment was less than that noted for the green mustard genotype.

3.6 Vanadium uptake

The addition of vanadium significantly ($p<0.001$) increased the vanadium concentration levels in the shoots and roots of the two mustard genotypes compared with the control treatment (Fig. 7). The increase in the vanadium concentration levels in the shoots of the green mustard genotype was 10.1, 31.2, 67.6 and 97.5 folds respectively, compared with the control treatment. This increase was estimated to 28.1, 52.7, 81.5 and 111.9 folds following application of 20, 40, 80 and 100 mg L^{-1} of vanadium, respectively in roots of green mustard genotype compared with the control treatment. Similarly, the vanadium concentrations were increased in the shoots and roots of the purple mustard genotype by 8.5, 29.7, 56.6 and 75.5 folds, and 43.6, 66.3, 100.7 and 133 folds compared with the control when treated with 20, 40, 80, and 100 mg L^{-1} of vanadium, respectively (Fig. 7). A significant difference was noted ($p<0.05$) for the mustard genotype interaction effect following vanadium uptake in the shoots and roots.

4 Discussion

We evaluated the effects of vanadium stress on growth and development of two mustard genotypes grown hydroponically by examining the morphological, physiological, and molecular changes in their leaves and roots. The leaves of both genotypes grown under vanadium stress gradually became shorter and a subsequent reduction in plant growth rate was observed compared to the plants grown under normal conditions in the absence of vanadium stress. At the initial stages of the experiment, the growth of the green genotype plants that were not treated with vanadium exceeded slightly that of the purple genotype leaves. One possible explanation for the changes noted in the growth of the plants following vanadium treatment could be due to reduced pigment synthesis in the leaves of both genotypes and due to a decrease in the efficiency

of photosynthesis by inhibiting the light capture by chlorophyll (Dixon et al., 2013). Moreover, a higher anthocyanin production might consume a certain amount of energy and nutrients of the plant cell and consequently disrupt the normal metabolic activities in the plant. The effects of vanadium stress on the growth of shoots and roots in the mustard genotypes were similar to those reported earlier (Rascio et al., 2008; Narumol et al., 2011; Imtiaz et al., 2015; Imtiaz et al., 2016; Imtiaz et al., 2017).

The net photosynthetic rate (*P*n) represents the ability of plants to produce CO_2 and is involved in various plant metabolic processes. The net photosynthetic rate is the rate of carbohydrate generated from the total photosynthesis minus the rate of respiration. The numerical value reflects the capacity of the plant to produce CO_2 (Yu-chen et al., 2015). The results of the current study demonstrated that vanadium stress negatively affected the photosynthetic apparatus of both mustard genotypes and with the increase in the intensity of vanadium stress, the net photosynthetic rate (*P*n), stomatal conductance (*g*s) and transpiration rate (*T*r) continued to decline rapidly. However, both mustard genotypes differed evidently in their sensitivity to vanadium stress as indicated by their differential response in the photosynthetic activity. These findings suggested that the photosynthetic apparatus and mechanisms were adversely affected by vanadium stress in mustard plants notably in the green genotype plants. These results provide some insights into the important role of light signaling to promote pigmentation in the leaves and the light-induced anthocyanin accumulation. The results of the present study are in accordance with some previous studies that have examined plant growth under a variety of metal stress conditions e.g. turf grass under Hg stress (Yu-chen et al., 2015), oilseed rape against Pb stress (Tian et al., 2014b), mustard grown under Cd stress (Mobin and Nafees, 2007), cucumber treated with metals (Burzyński and Kłobus, 2004) and *Artemisia annua* under Zn stress conditions (Khudsar et al., 2004).

Metal toxicity regulates reactive oxygen species (ROS) by inducing irreversible oxidative damage to various macromolecules (Jain et al., 2010). The plants initiate an antioxidant defense

system consisting of specific enzymes in order to scavenge the metal stress. Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are the integral components of the plant enzymatic mechanism required to scavenge ROS. These enzymes can be used as biomarkers for environmental stress (Jain et al., 2010).

The enzymes that exist in various cellular compartments, are the key parts of the anti-oxidant defense system in plants that regulate the ROS. Previous studies have reported higher anti-oxidant enzyme activities in plants as a mechanism to restore the oxidative damage (Dazy et al., 2009). The results of the current study exhibited a positive relationship between vanadium concentrations and enzymatic activities. The significant increase in enzymatic activities of SOD, CAT, and POD were observed in response to the increase in vanadium concentration levels. However, the enzymatic activity of these enzymes was higher in the shoots of the purple mustard compared with the corresponding activity in the shoots of the green mustard genotype. In addition, vanadium concentrations of 80 and 100 mg L^{-1} resulted in a pronounced increase in these enzyme activities compared with the control treatment. This is probably due to the generations of additional levels of ROS that result from increased oxidative damage caused by NH_4VO_3. Ke and coworkers demonstrated that higher concentrations of Cu caused increased enzymatic activities in turfgrass (Ke et al., 2007). The increase in SOD, CAT and POD enzymatic activity levels could be due to the direct effect of heavy metal ions and the indirect effect on the production of free oxide radicals (Zembala et al., 2010). The up-regulation of enzyme activities could be attributed to de novo synthesis of enzyme proteins, whereas the high activity of CAT enables the plant to eliminate the H_2O_2 by conversion to O_2 and H_2O. The SOD activity regulates the superoxide radicals and yields water and O_2, thus retaining the superoxide radicals to a steady state. The high POD activity protects the cellular membrane against the oxidative damage from the metal stress and further enables the plants to scavenge H_2O_2 in cells (Verma and Dubey, 2003; Miller et al., 2008; Monferrán et al., 2009). The data reported in the present study are in general agreement with the aforementioned studies and are supported by

previous findings (Tanyolaç et al., 2007; Miller et al., 2008; Gupta et al., 2009; Jain et al., 2010; Imtiaz et al., 2015; Imtiaz et al., 2017).

The accumulation of anthocyanin molecules in the plant organs, such as the leaves and shoots is a common phenomenon under abiotic stress that can occur in the presence of heavy metals. Therefore, the anthocyanin production is a part of the defense system in plants in stressed environments (Merzlyak et al., 2008). In the present study, the anthocyanin contents were reduced significantly in both green and purple mustard plants following exposure to vanadium stress. However, the green mustard plants exhibited lower anthoycyanin contents compared with the purple mustard. We found that the response of the purple plants for the anthocyanin contents was more or less similar at the intermediate vanadium stress treatment of 20 and 40 mg L^{-1}; however, at the higher vanadium concentration levels of 80 and 100 mg L^{-1} the anthocyanin contents were severely inhibited. The decrease in the anthocyanin production at higher vanadium concentrations accompanied by a simultaneous reduction in the photosynthesis activity (Dai et al., 2006). The biological and antioxidant activities of the phenolic compounds in plants have been studied extensively under metal stress conditions (Wang and Lin, 2000; Tsai et al., 2002). In addition, the role of anthocyanin production in *Brassica* species has been reported earlier (Glin´ska et al., 2007). Furthermore, ROS such as H_2O_2 diffuse rapidly through cell membranes and can allow vascular anthocyanins to scavenge ROS (Hatier and Gould, 2009). The present study further suggested that the biosynthesis of anthocyanins could be an effective strategy against oxidative damage caused by vanadium stress for at least 40 mg L^{-1} of vanadium concentration levels. Similar results have been observed for the stress induced by Cd and Ni, where anthocyanin contents in the plants remained at high levels following treatment with low concentrations of these metals, whereas they were inhibited significantly when the plants were exposed to higher metal concentrations (Hawrylak et al., 2007; Zhang et al., 2014).

We studied the expressions of anthocyanin biosynthetic genes and few transcriptional factors in the leaves of both mustard genotypes in order to evaluate the association between anthocyanin

accumulation and the expression of anthocyanin biosynthetic genes. Our results showed that adequate expressions of related anthocyanin biosynthetic genes were essential for an appropriate anthocyanin biosynthesis. As a result, the expression profiles of these anthocyanin biosynthetic and regulatory genes correlated with the patterns of anthocyanin accumulation in the leaves of the purple mustard plants. Therefore, the increased expression of PAL2, PAL4, C4H, 4CL, CHS, CHI, F3H, TT8, ANS, DFR, UGTs, MYBL2 and TT19 genes was observed that could activate the production of visible pigmentation in the seedlings of the purple mustard genotype. Higher expression levels of anthocyanin biosynthetic genes were noted in the vanadium treated plants, whereas the gene expression levels in the purple mustard genotype were also higher compared with the green genotype plants. The expression levels of these genes were the highest in the purple mustard leaves at 20 and 40 mg L^{-1} of vanadium treatment. The expression of the TT8 and F3H genes was highly induced for the purple mustard genotypes even at 100 mg L^{-1} of vanadium concentration. The results were consistent with their phenotype and further illustrated that the expression of the structural genes was closely related to the anthocyanin accumulation in the leaves of the purple mustard plants.

The anthocyanin biosynthetic genes are largely regulated at the transcriptional level via a complex of MYB, bHLH transcription factors and WD-repeat proteins (Baudry et al., 2004). The regulators interact with each other to form transcriptional complexes, which bind to the promoters of the structural genes in order to activate transcription (Sainz et al., 1997; Koes et al., 2005). The present study suggested that the induction of TT8 expression was likely to activate the expression of the structural genes (F3H, F3′H, DFR, and ANS) of the anthocyanin biosynthesis, and therefore, TT8 is likely to be a critical gene required to control the anthocyanin biosynthetic pathway in the purple mustard under vanadium stress. This is probably the general mechanism underlying anthocyanin production in the leaves of purple mustard. This regulatory mechanism is also similar to that reported in the red cabbage (Yuan et al., 2009).

Protein production in plants is considered sensitive to heavy metal stress and it declines under metal toxicity (Hemalatha et al., 1997). In the present study, the protein contents further demonstrated a negative relationship with vanadium treatment. The protein contents were decreased at all vanadium treatments in the mustard plants, although the more pronounced effects were observed at 80 and 100 mg L^{-1} of vanadium concentrations compared with the control treatment. Heavy metals generally damage the functional ability of a large number of enzymes that contain functional sulphydryl groups and ultimately affect the protein production (Tanyolaç et al., 2007). A study conducted by Andon and Fernando (2011) highlighted that high levels of cadmium inhibited the protein production in barley plants. The current findings are consistent with the study by Andon and Fernando and various other studies (Zhou et al., 1998; Guo et al., 2004; Tanyolaç et al., 2007; Imtiaz et al., 2017).

The absorption and accumulation of vanadium in shoots and roots of both mustard genotypes were increased with increasing concentrations of vanadium. It has also been reported that vanadium is unevenly distributed in tissues of plants and roots. This uneven distribution ultimately limits the vanadium transportation to shoots (Imtiaz et al., 2015) as shown in the present study. The results indicated that the green mustard genotype retained less vanadium than the purple mustard plant. In general, roots accumulated more vanadium than shoots in both mustard genotypes. However, the roots of the purple mustard genotypes accumulated more vanadium than the roots of the green mustard plants. Similarly, higher vanadium uptake was reported in the roots for the Chinese green mustard, the tomato plants treated with NH_4VO_3 (Narumol, et al., 2011), the rice plants and the chickpea plants (Rascio et al., 2008; Imtiaz et al., 2015; Imtiaz et al., 2017).

Conclusions

In conclusion, the toxicity of vanadium significantly decreased the photosynthetic processes, and markedly increased the activities of antioxidant enzymes. However, relatively greater tolerance

of the purple mustard genotype to vanadium stress might indicate optimal synergies between the enzymatic activities of the antioxidant enzymes in order to protect the plant photosynthetic apparatus. The present study further indicated a negative correlation between vanadium concentrations and anthocyanin biosynthesis in both mustard genotypes. This correlation was more evident in the purple mustard plants and suggested that the up-regulation of TT8, F3H and MYBL2 could trigger the accumulation of anthocyanins under vanadium stress. Therefore, TT8, F3H, and MYBL2 genes could be key genes that control the anthocyanin accumulation in the purple mustard genotype under vanadium stress. Further studies are required for genetic analysis, and in order to clarify the role of TT8, F3H, and MYBL2 along with MYB and WD genes in the production of anthocyanins in the purple mustard genotype under vanadium stress conditions.

COMPETING INTERESTS

None of the authors have any competing interests.

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Figure legends

Fig. 1. Effect of vanadium doses on the growth and appearance of hydroponically grown mustard genotypes that differ in photosynthetic capacity: green (higher photosynthetic capacity) and purple (lower photosynthetic capacity).

Fig. 2 Effect of vanadium doses on net photosynthetic rate (A), stomatal conductance (B) and transpiration rate (C) of mustard genotypes that differ in photosynthetic capacity: green (higher photosynthetic capacity) and purple (lower photosynthetic capacity) grown hydroponically. Values represent mean±S.D. (n=3).

Fig. 3 Effect of vanadium doses on the activity of antioxidant enzymes (A) Superoxide dismutase (B) (SOD), Catalase (CAT) and (C) Peroxidase (POD) of mustard genotypes that differ in photosynthetic capacity: green (higher photosynthetic capacity) and purple (lower photosynthetic capacity) grown hydroponically. Values represent mean±S.D. (n=3)

Fig. 4 Effect of vanadium doses on anthocyanin accumulation in the leaves of green and purple mustard genotypes. Values represent mean ± S.D. (n=3)

Fig. 5 Relative expression of 17 anthocyanin biosynthesis genes in two mustard genotypes that differ in photosynthetic capacity: "G" denotes green genotype (higher photosynthetic capacity) and "P" denotes purple genotype (lower photosynthetic capacity) grown hydroponically under vanadium stress. Total cellular RNA was extracted from leaves following 30 d of treatment and was subjected to semi qPCR using specific primers against each receptor subtype. Control: zero V; T1 V@ 20 mg L^{-1}; T2 V@ 40 mg L^{-1}; T3 V@ 80 mg L^{-1} and T4 V@ 100 mg L^{-1}. The relative expression of all the genes was normalized with β-actin.

Fig. 6 Effects of vanadium doses on soluble protein contents of mustard genotypes that differ in photosynthetic capacity: green (higher photosynthetic capacity) and purple (lower photosynthetic capacity) grown hydroponically. Values represent mean ± S.D. (n=3)

Fig. 7 Vanadium concentration of mustard genotypes that differ in photosynthetic capacity: green (higher photosynthetic capacity) and purple (lower photosynthetic capacity) grown hydroponically. Values represent mean ± S.D. (n=3)

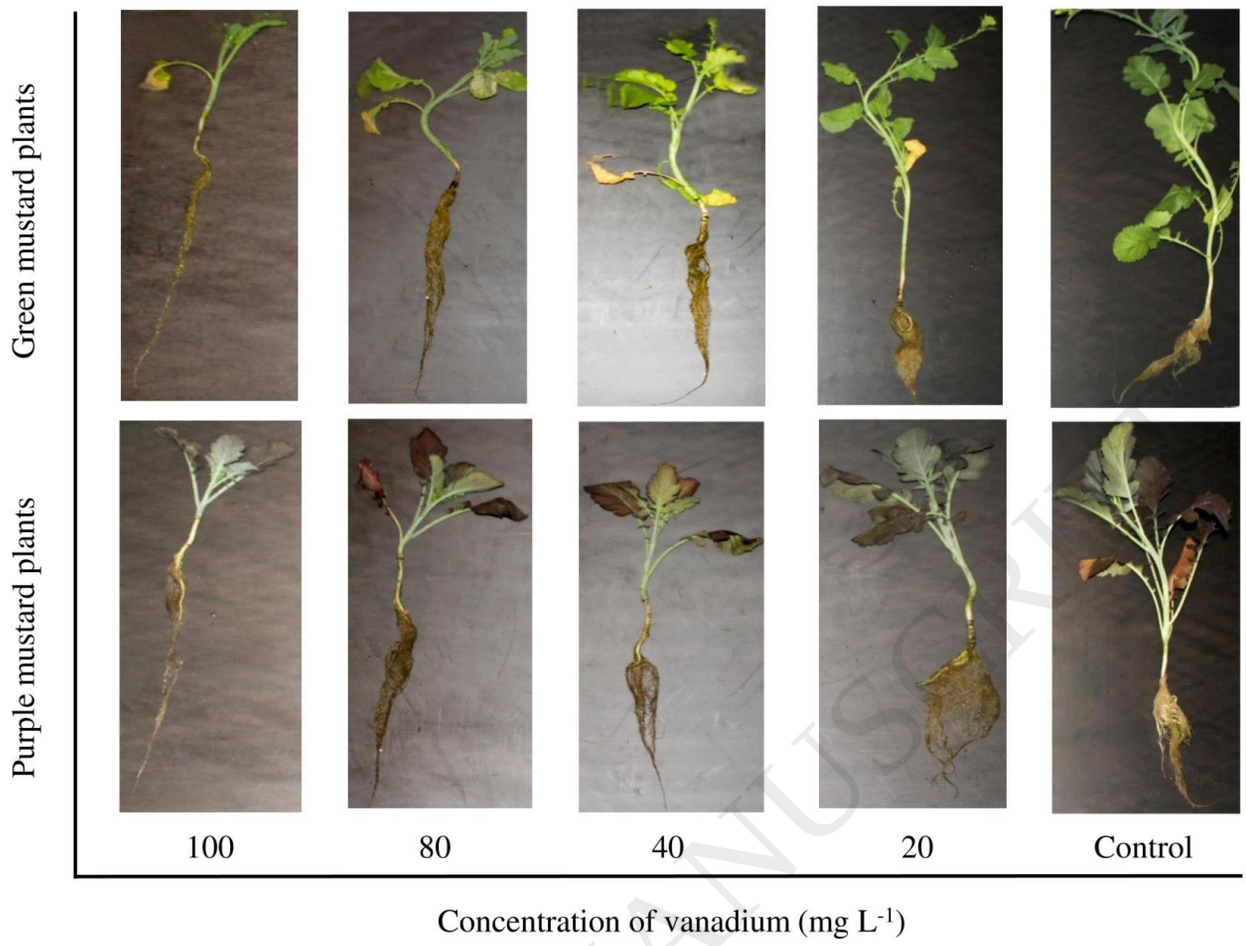

Figure 1.

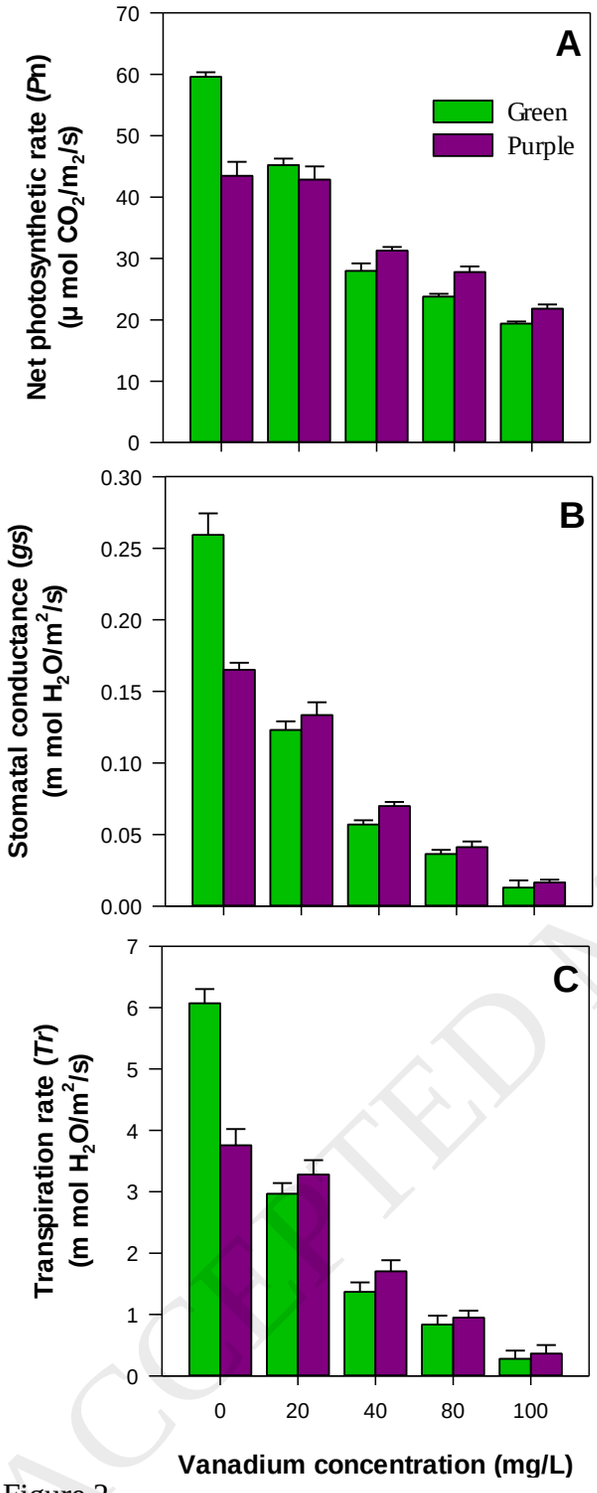

Figure 2.

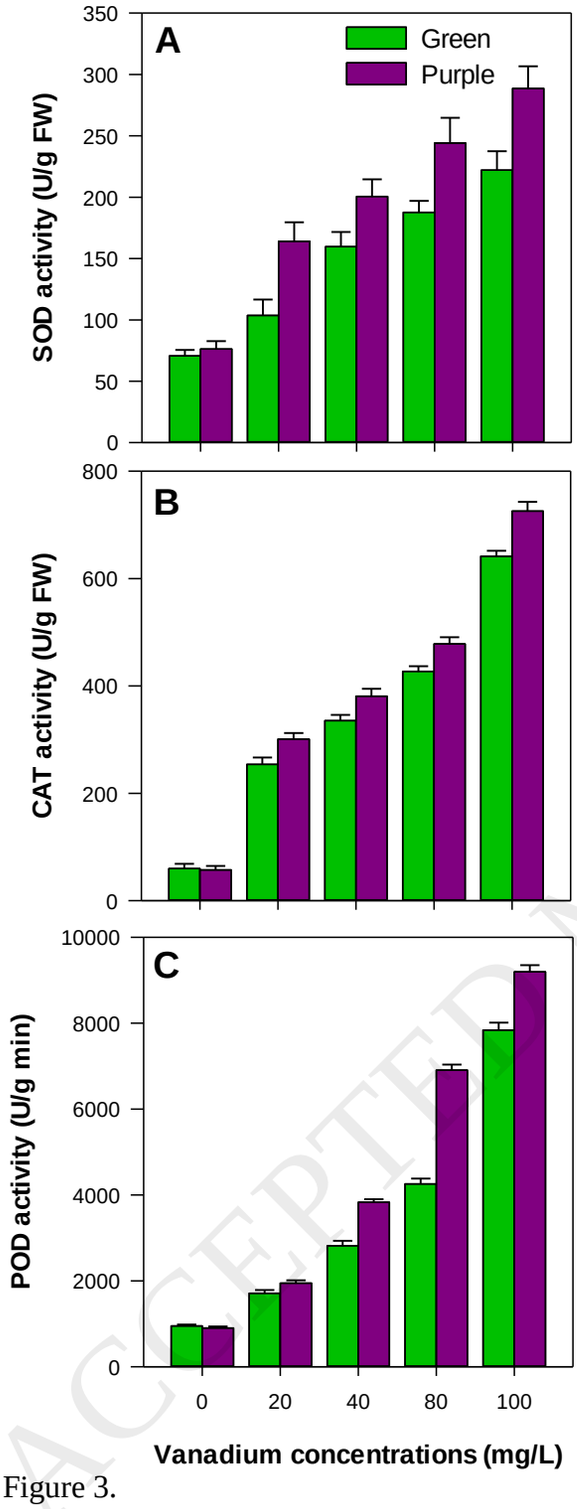

Figure 3.

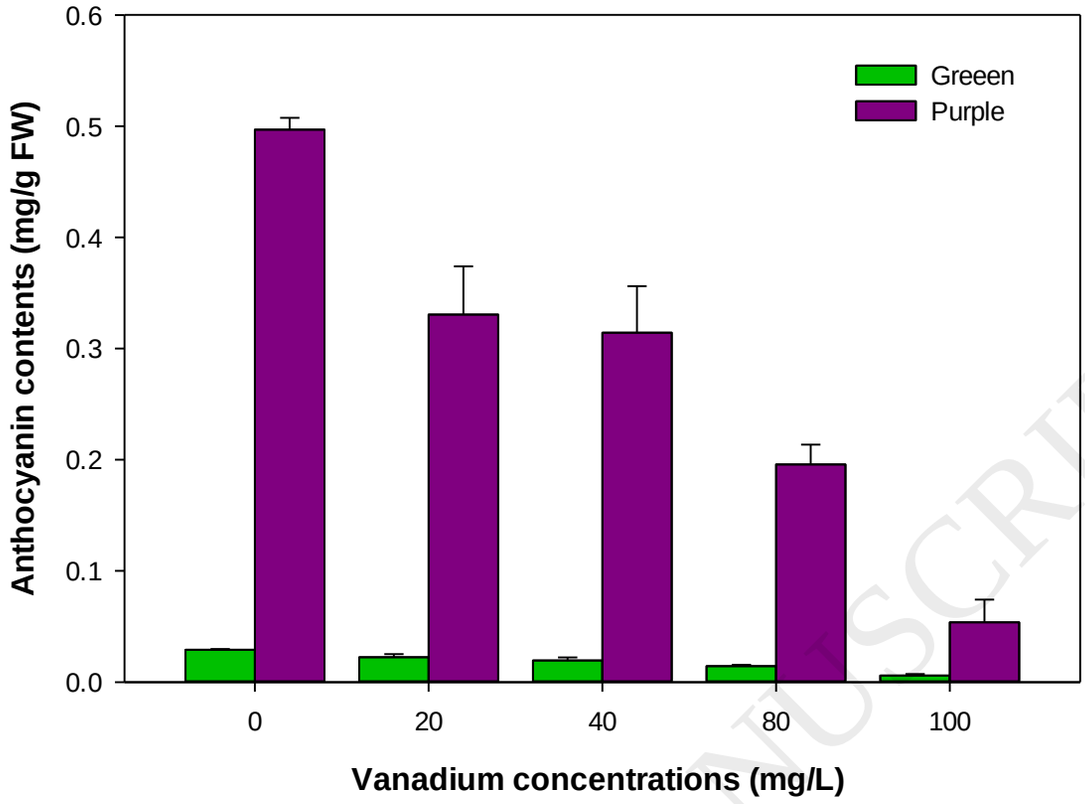

Figure 4.

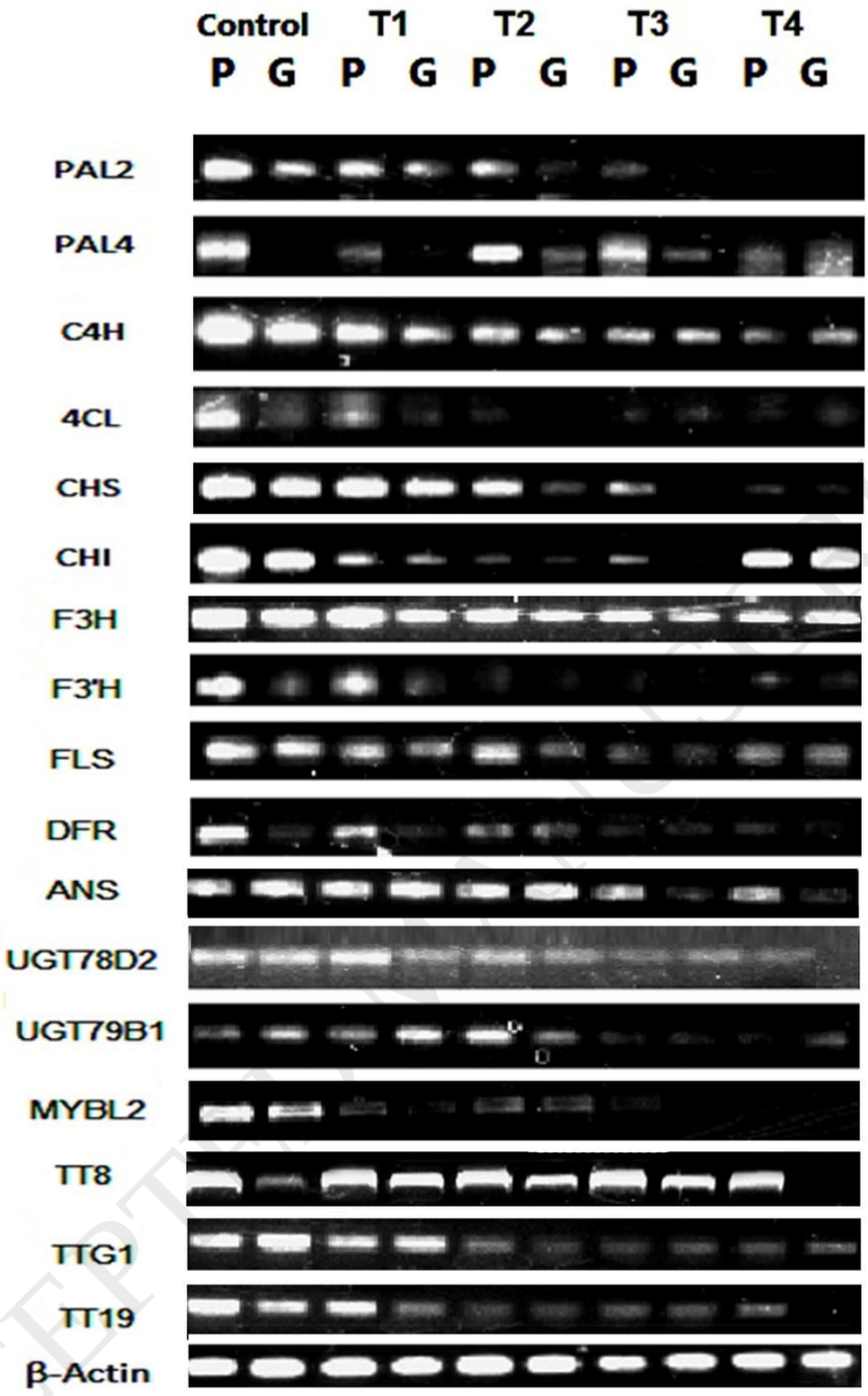

Figure 5.

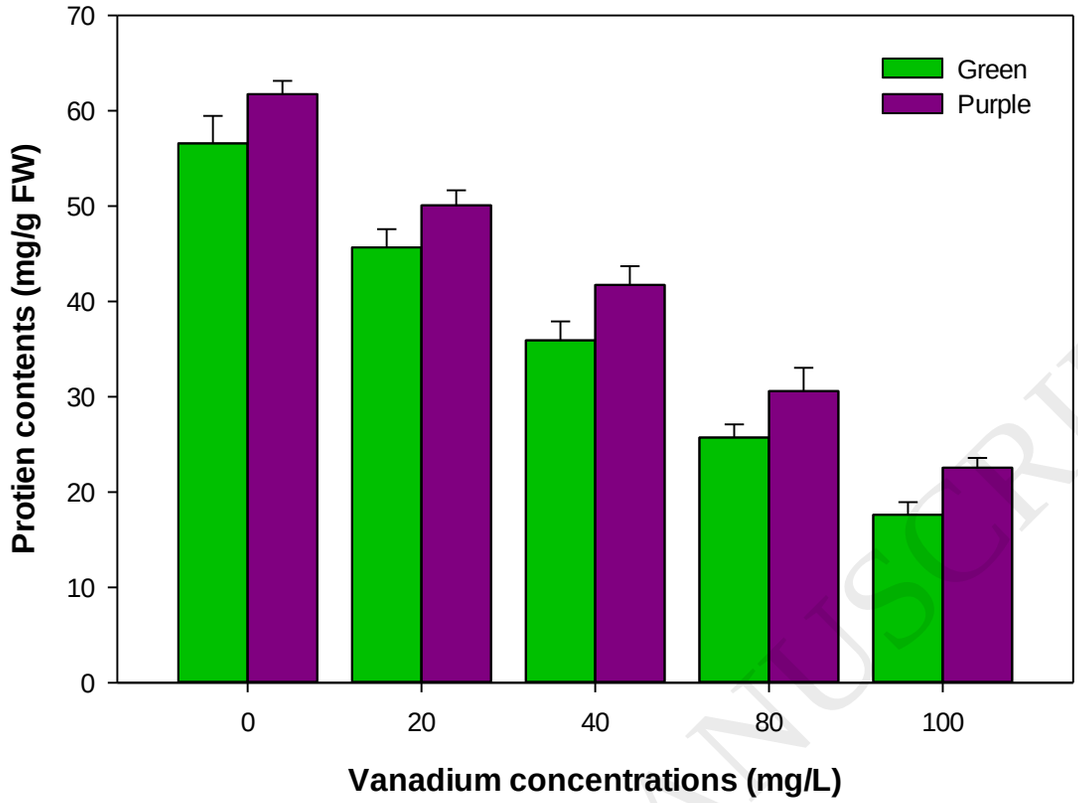

Figure 6.

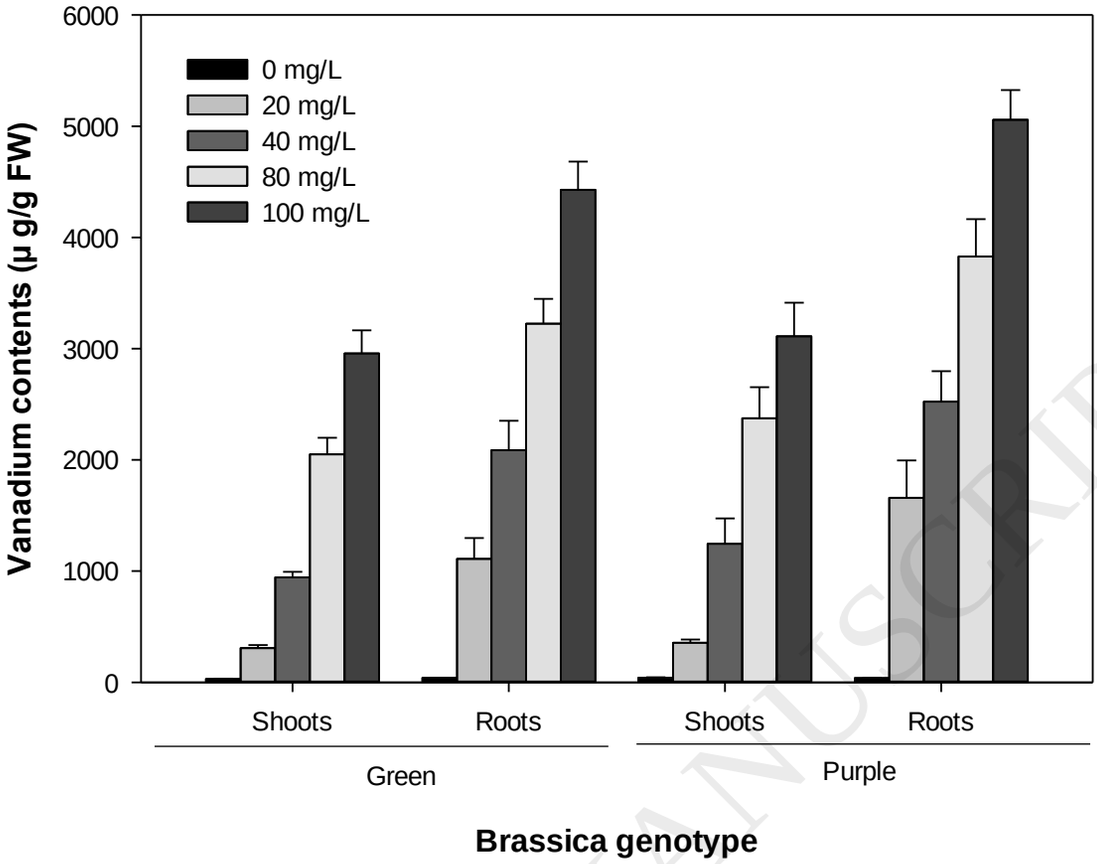

Figure 7.

Table 1
Primers used for semi quantitative RT-PCR analysis

Gene	Gene bank ID	5' to 3' Forward Primer	5' to 3' Reverse Primer
PAL2	AT3G53260	CGACGGAAGCTCGTACATGA	CCACTGAGGAGAGGTACGGA
PAL4	AT3G10340	CCTCTCTTACATCGCCGGAC	AACAGAACCATCGAGGCCAG
C4H	AT2G30490	GTCGCTGCGATTGAGACAAC	TCAGGCTCTGTGACTTGCAC
4CL	AT3G21230	CGATCTTGATCGTTCCGAGG	GCCTGAAAGCATCATCCTCA
CHS	AT5G13930	GCGATCCTTGACGAGGTTGA	CGTCGTAGCCACACCATCTT
CHI	AT3G55120	TGACGATGAAGCTGCCGTTA	AGCGAAGAGGATGGATGCAC
F3H	AT3G51240	CCTGGAACCATCACTTTGCT	CCGTTGCTCAGATAGTGACC
F3’H	AT5G07990	AAGAGCTTGATTCCGTCGTC	CCGTTGATCTCACAGCTCTC
FLS	AT5G63600	AGGCGATCCGATCTGGAGAA	CCATCTCCTGTGTGCTTCGT
DFR	AT5G42800	AGCCTTATTACCGCGCTCTC	TTGGCAGCAGCTTGTTCGTA
ANS	AT4G22880	AGCCTGACCGTCTAGAGAAA	GACCAGGAACCATGTTGTGT
UGT78D2	AT5G17050	AGGACTGCTTCTGTAGCGTA	CTTGCTCTCTTGTCCGTTCA
UGT79B1	AT5G54060	GATGGTTTTGGTTCCGCAGC	CCATCACACTCTTCACGGCA
TT8	AT4G09820	TAAGGCGGTGGTGCAATCTG	CTTGTTCGTTGTGCCTAGTTCC
TTG1	AT5G24520	ATCTTCGATCTGCGCGACAA	CGTGGCCATACACCTCAAGT
MYBL2	AT1G71030	TCGATCCAACCAATCATCGT	CCGGCACTAGCATTATCCTC
TT19	AT5G17220	CCAAGGCACGAACCTTTTGG	GCCTAACCTCGGCTTGATGA

Table 2
Multivariate analysis of variance (MANOVA) to test the effects of vanadium concentration and genotype of plant variables

Source	Dependent Variable	df	F-value	Significance
Vanadium concentration	Net photosynthetic rate	4	59.21	***
	Stomatal conductance	4	83.97	***
	Transpiration rate	4	84.11	***
	SOD activity	4	188.1	***
	CAT activity	4	2648	***
	POD activity	4	46.62	***
	Anthocyanin content	4	37.25	***
	Protein content	4	162.4	***
	Vanadium contents in shoot	4	579.6	***
	Vanadium contents in roots	4	445.1	***
Genotype	Net photosynthetic rate	1	3.108	*
	Stomatal conductance	1	3.803	*
	Transpiration rate	1	3.687	*
	SOD activity	1	102.5	***
	CAT activity	1	109.8	***
	POD activity	1	14.82	**
	Anthocyanin content	1	421.8	***
	Protein content	1	8.542	**
	Vanadium contents in shoot	1	21.09	***
	Vanadium contents in roots	1	26.29	***
Vanadium Concentrations × Genotype	Net photosynthetic rate	4	5.049	**
	Stomatal conductance	4	6.407	**
	Transpiration rate	4	7.330	**
	SOD activity	4	4.533	**
	CAT activity	4	12.59	***
	POD activity	4	1.437	ns
	Anthocyanin content	4	30.21	***
	Protein content	4	3.283	*
	Vanadium contents in shoot	4	3.727	*
	Vanadium contents in roots	4	2.342	*

ns = non-significant; * = $p<0.05$; ** = $p<0.01$; ***= $p<0.001$