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REGULATION OF DIFFERENT INTENSITIES OF UV-B IRRADIATION ON PHENOTYPIC DEVELOPMENT OF ALFALFA RADICLE

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Abstract

Roots are one of the important organs in a plant. Under different intensities of ultraviolet-B (UV-B) irradiation condition, plant roots have displayed different responsive behaviors at multiple levels of physiological, biochemical and cellular metabolisms. The higher intensities of UV-B have induced more damages to alfalfa roots than the lower intensities of UV-B irradiation, including biomass reduction, ROS toxicity, cell vitality decrease, and microstructure abnormality and physiochemical component alteration. The experimental data in our studies have shown that antioxidant/pro-oxidant balance play an important role in UV-B tolerability of plant root systems, which would be closely related to the UV-B protection action of plant proteins (FTIR wave number 1700-1600cm^{-1} and 1300-1200cm^{-1}), cell membrane lipids and cell wall pectins (1745cm^{-1}).

Key words: Alfalfa, Ultraviolet-B, Radicle, ROS toxicity, Antioxidant/pro-oxidant.

Introduction

UV-B (wavelength 280-320nm) is the most energetic component of sunlight spectrum, and has extensive impacts on the whole biosphere (Jenkins, 2009; Yin and Ulm, 2017). As an informational signal from natural environments, UV-B irradiation has direct effects not only on diverse organisms, but also on the complex ecosystems (Jenkins, 2009; Yin and Ulm, 2017; Yao *et al*., 2021). Previous works have shown that the suitable dosage of UV-B irradiation, as a positive factor, regulates plant photomorphogenesis and phenotypic responses, such as organ and tissue development, biochemical metabolism, reactive oxygen species (ROS) equilibrium, UV-B-responsive gene expression, UV-B protection and UV-B acclimation pathways (Ulm & Nagy, 2005; Gao *et al*., 2019a). While the higher intensities of UV-B would be a potential stressor to inhibit plant growth and development, and the long-time exposure of UV-B stressor might significantly alter the nutritional ingredients in plants and decrease its germplasm yield (Ulm & Nagy, 2005; Li *et al*., 2016; Liang *et al*., 2019; Gao *et al*., 2019a). In view of this, the biological effects of different intensities of UV-B irradiation from sunlight on plants are entirely distinct. Roots are one of the most important organs for higher plants. Root systems can transport water molecules, minerals and other nutrients for plant growth and development. Thus, it is responsible to investigate root responsive behaviors to environment factors, such as UV-B irradiation, for exploring interactive mechanism between plant cell and environment.

Our previous experiments have investigated alfalfa seedling responses to different intensities of UV-B irradiation in details, and we found that alfalfa seedlings have higher resistance to low intensity UV-B radiation (0 μWcm^{-2} day^{-1}<UV-B radiation<17.35 μWcm^{-2} day^{-1}). But high intensity UV-B radiation (UV-B radiation>17.35 μWcm^{-2} day^{-1}) have resulted to significant damages for alfalfa seedlings in many aspects of growth rate, phenotypic development, physiological processes, biochemical metabolisms and gene transcriptional activities (Gao *et al*., 2019b). Xie *et al*. have also reported that alfalfa seedlings were subjected to varying degrees of stress reactions with the increase of UV-B from 6.0 KJ m^{-2} to 13.2 KJ m^{-2} (Xie *et al*., 2015). With respect to the controls, fresh weight and chlorophyll content of alfalfa seedlings gradually reduced, while thiobarbituric acid-reactive substances remarkably increased with the increment of UV-B (Xie *et al*., 2015). However, these data are only limited to the aerial parts of a plant under UV-B irradiation growth conditions. There are few reports on the bioeffects of UV-B irradiation on plant root systems.

It is well established that plant roots are also involved in many biochemical processes to light stimulation, including UV-B irradiation (Feng *et al*., 2003; Ghosh & Xu, 2014). Many photoreceptors, such as UV-B photoreceptor UVR8, have existed in both of aboveground part of a plant and its underground root systems (Ghosh & Xu, 2014; Rafińska *et al*., 2017; Yang *et al*., 2018). Intracellular ROS accumulation has also been speculated to be related to the responses of plant roots to UV-B (Feng *et al*., 2003; Comont *et al*., 2013; Jenkins, 2014). However, it is still unknown how UV-B irradiation leads to intracellular ROS accumulation in root tissues, and root cells are mediated to perceive UV-B through which signaling pathways. It is worth mentioning that the pectin fraction and membrane lipid in cell wall might play an important role in plant root response to UV-B irradiation (Zhong & Lauchli, 1993; Feng *et al*., 2003; Ghosh and Xu, 2014; Jenkins, 2014; Yin *et al*., 2016). To date, the information on these ways is also very limited. Hence, the further studies on the responses of plant root systems to different intensities of UV-B will provide abundant data for further solving these problems. Fourier transform infrared (FTIR) have widely used in structural analysis of macromolecular compounds, identification of cell wall mutants, and secondary structural analysis of proteins (Wilson *et al*., 2000; Yang *et al*., 2002; Mouille *et al*., 2003). The infrared spectra of higher plant leaf have similar absorption characteristic peaks. The peak of

1745cm^{-1} represents vibration region of COOR functional groups, which belongs to cell wall esters including cell membrane lipid and cell wall pectins (Wilson *et al*., 2000). The 2000-1000cm^{-1} absorption intervals of plant leaf display three amide regions of proteins, such as 1700-1600cm^{-1} for NH_2-I, 1600-1500cm^{-1} for NH_2-II, and 1300-1200cm^{-1} for NH_2-III (Yang *et al*., 2002). NH_2-I region is the sensitive absorption spectra, thus, we primarily analyzed relative peak areas of 1700-1600cm^{-1} for NH_2-I to test protein contents.

We have examined phenotypic development and biochemical metabolism changes in alfalfa radicle, including growth parameters, ROS accumulation, cell vitality, plant root protein and cell wall esters physiochemical character under different intensities of UV-B conditions in our works. And then the microstructures of alfalfa root systems have been further tested. The primary objective is to deeply explore responses of plant root systems to different intensities of UV-B irradiation and its probable mechanisms.

Materials and Methods

Plant materials and treatments: Mature seeds of alfalfa are incubated on Petri dishes with wet filter papers for germination under 26°C, dark conditions. After two days, seeds with consistent germination times were selected at 22°C for UV-B treatment. The germinated seeds were exposured with four different intensities of UV-B, including very low flux (VLF), low flux (LF), medium flux (MF) or high flux (HF) according to our previous methods (Gao *et al*., 2019b). The controls were also cultured under normal conditions without UV-B irradiation at 22°C. For all groups, the white light intensity is 100μmol m^{-2} s^{-1}, with photoperiod 10/14h light/dark cycle (Gao *et al*., 2019b) (Fig. 1a). Alfalfa radicles were exposured for 8h d^{-1} with UV-B for 1d, 2d or 3d, respectively, and then collected for further analysis.

Growth changes of alfalfa roots: On the second day after UV-B treatment, the growth parameters of alfalfa radicles were tested. FW (fresh weight) values were determined with fresh root tissues by Ultramicro Electronic Balance (Sartorius, Germany), and DW (dry weight) data were determined after drying the roots at 80°C in an oven for 48h (Gao *et al*., 2019b). The phenotypic characters of alfalfa radicles were analyzed.

ROS detection: The ROS (superoxide anion $O_2^{\cdot-}$ and hydrogen peroxide H_2O_2) accumulation was visualized in root tissues with NBT (nitroblue tetrazolium, 6.0mM) or DAB (3,3'-diaminobenzidine tetrahydrochloride, 1.0mg ml^{-1}) according to the previous reported methods (Xie *et al*., 2015; Cembrowska-Lech *et al*., 2015).

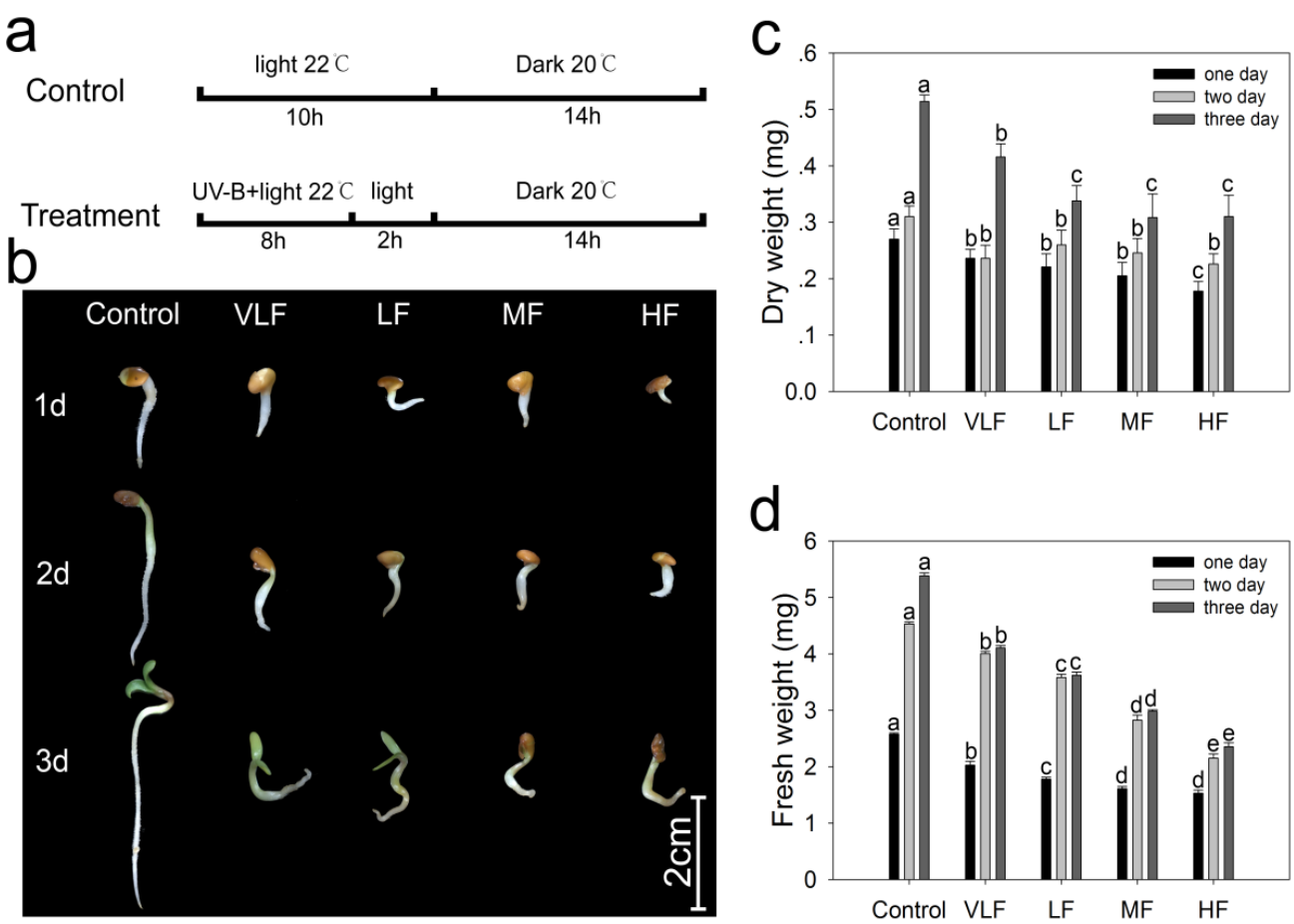

Fig. 1. Effects of UV-B irradiation on alfalfa radicle growth and development.
a. Growth conditions of plant materials; b. Phenotype of plant radicles; c. Dry weight values of plant radicles; d, Fresh weight values of plant radicles. Data are shown as means±s.e. (n=6). VLF, very low flux of UV-B irradiation; LF, low flux of UV-B irradiation; MF, medium flux of UV-B irradiation; HF, high flux of UV-B irradiation.

Cell vitality analysis: The vitality of root-tip cells from different growth conditions was analyzed by Calcein-AM/PI Kit (Kanglang Biotechnology co. LTD, Shanghai City, China) according to the instructions. Living cells can be stained by Calcein-AM fluorescence dye to display green fluorescence, while the inactive cells are labeled with red fluorescence by Propidium Iodide (PI) dye.

Root microstructure observation: Root tissues from various treatments were collected and prepared for microscopic observation by ultrathin sections (Leica, EM UC6) (Wang *et al*., 2013). Samples were dyed with toluidine blue-O for 3-5min and observed under optical microscopes (Japan, Olympus, BX53).

Cell protein and cell wall esters physiochemical properties: Cell esters are composed with cell wall pectins and membrane lipid. The radicles of alfalfa were collected and dried quickly for 2d under 55°C to eliminate water molecules. Physiochemical properties of the total proteins and cell wall esters were detected by FTIR (Fourier transform infrared) scanning spectra (USA, Varian 660-IR). FTIR spectra were obtained from wavelength 4000-400cm^{-1} (2cm^{-1} of resolution ratio, 32 scanning times) at room temperature. The relative contents of plant proteins (1700-1600cm^{-1}) and cell wall esters (1745cm^{-1}), and the relative percentage changes of protein secondary structure (1700-1600cm^{-1} and 1300-1200cm^{-1}) have been analyzed.

Results

Growth and development of alfalfa radicles: Under UV-B irradiation, the growth and development of alfalfa radicles were inhibited, and these inhibitory effects became more significant with the increment of UV-B intensity or the extension of exposure time. Comparing to controls, plant radicles exhibited a shortened, irregular curly phenotype in UV-B irradiated-plants (Fig. 1b). In addition, the growth parameters, such as FW and DW had been greatly altered in UV-B treated groups. Furthermore, these changes would be more remarkable with the increment of UV-B irradiation dosage and the extension of UV-B duration (Fig. 1c, d).

ROS levels: Histochemical staining was performed to test the intracellular ROS accumulation in radicles under different growth conditions. We found that UV-B irradiation has brought about brown (DAB staining) or dark blue (NBT staining) color reactions, suggesting the more H_2O_2 and $O_2^{\cdot-}$ accumulation in root tissues compared to controls. However, alfalfa roots had faced less oxidative stress under the lower intensities UV-B than those of the higher intensities UV-B radiation (Figs. 2 and 3).

Cell vitality: The results of root-tip cell vitality had also displayed that the higher intensities UV-B irradiation caused the loss of cell vitality compared to controls under normal growth conditions (Fig. 4a). Whereas, root-tip cell displayed the higher vitality under the lower intensities of UV-B growth conditions (Fig. 4b). The double staining results were consistent with the above single staining results (Fig. 4c).

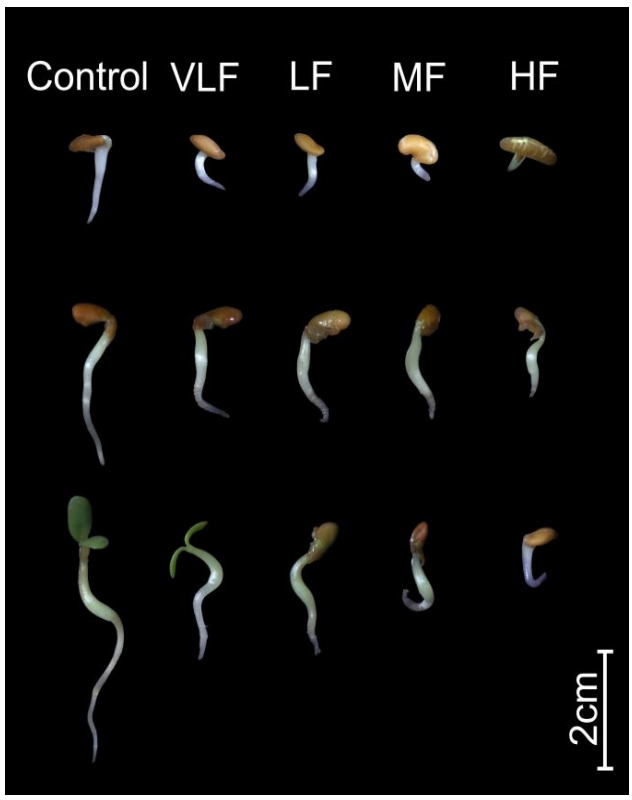

Fig. 2. NBT histochemical staining of plant radicles.
VLF, very low flux of UV-B irradiation; LF, low flux of UV-B irradiation; MF, medium flux of UV-B irradiation; HF, high flux of UV-B irradiation. Bar=2cm.

Fig. 3. DAB histochemical staining of plant radicles.
VLF, very low flux of UV-B irradiation; LF, low flux of UV-B irradiation; MF, medium flux of UV-B irradiation; HF, high flux of UV-B irradiation. Bar=2cm.

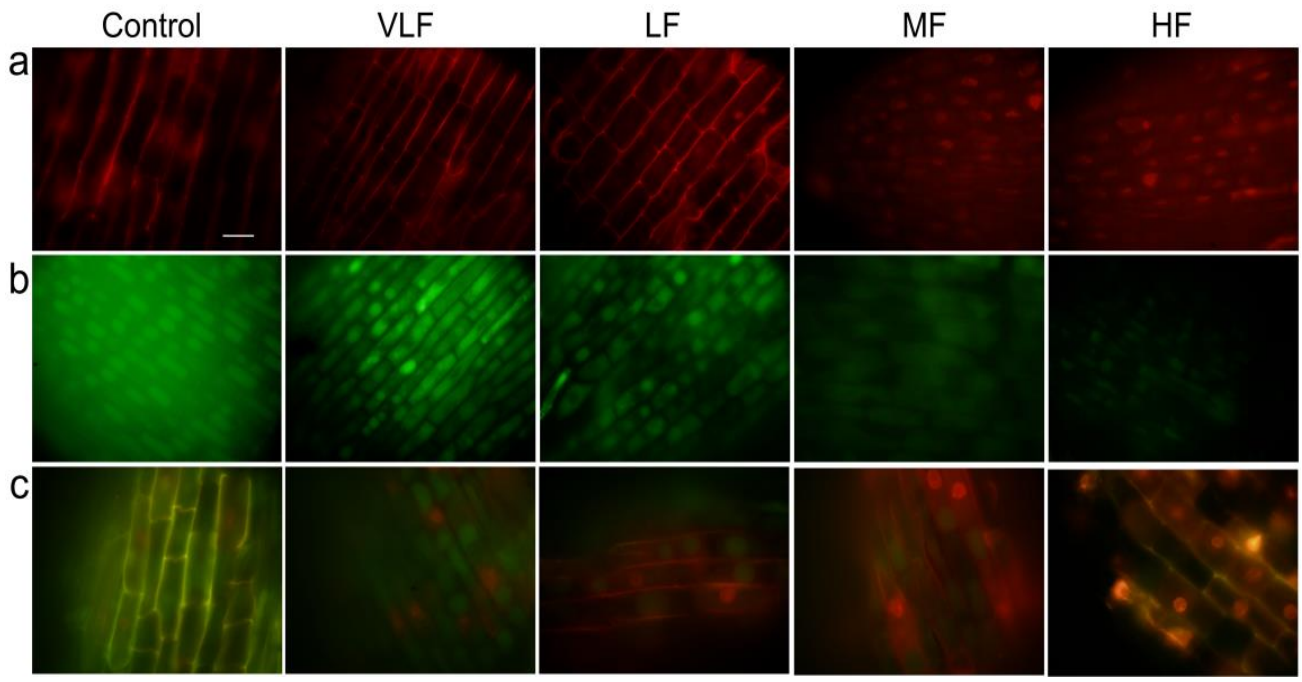

Fig. 4. Cell vitality detections of plant root-tip cells.
a. Single dyeing with PI; b. Single dyeing with Calcein-AM; c. Double dyeing with PI and Calcein-AM. Bar=50μm.

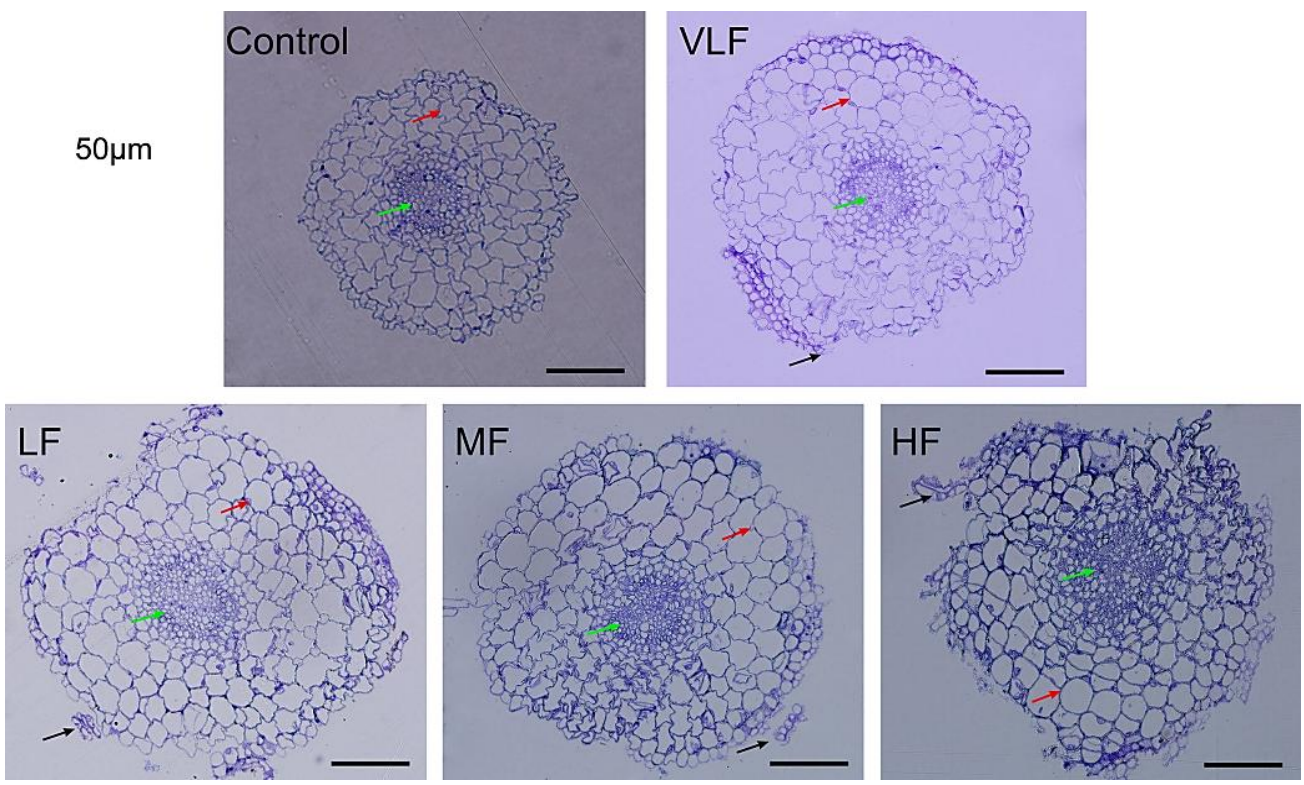

Fig. 5. Microstructure of plant radicles.
Black arrows, epidermal cells and cortical cells; green arrows, xylem ridge; red arrows, the shape of cells. Bar=20μm.

Radicle microstructure observation: Compared with controls, UV-B irradiation altered the microstructure of radicle tissues. UV-B irradiation led to the shedding of epidermal cells and cortical cells in radicles (black arrows), but the diameter of radicles become thicker. Control radicles showed three bundles of radically arranged xylem ridge. Exposure to UV-B irradiation, the arrangement of xylem ridge was disordered (green arrows). Meantime, cortical cells are irregular in control radicles, while UV-B treatment caused cortical cells to change into round or oval (red arrows) (Fig. 5).

Cell wall ester and protein physicochemical properties: Cell wall ester and protein compounds of

alfalfa radicles were also determined by FTIR spectra. We have found that UV-B irradiation resulted to the changes of characteristic peak areas of cell wall ester ($1745cm^{-1}$) and amide I ($1700\text{-}1600cm^{-1}$) of proteins FTIR absorption. Furthermore, cell wall ester and protein contents have also altered compared with controls. Exposure to UV-B irradiation for 1d, their contents would increase following UV-B intensities. While being after UV-B treatment for 2d or 3d, their contents would significantly decrease with the increment of UV-B irradiation (Fig. 6). After UV-B stress 3d, the relative abundance of protein secondary structure had no obvious changes in VLF and LF groups, while significant or highly significant alterations in MF or HF groups (Fig. 7).

Discussions

UV-B, an intrinsic part of sunlight spectrum, is one of important environmental factors for the survival of organisms, especially for higher plants due to its sessile growth pattern (Ma *et al*., 2018; Liang *et al*., 2019; Gao *et al*., 2019b). UV-B irradiation has dual regulation effects on plant growth and development, which not only serves as a positive signal to promote plant phenotypic responses, but also is a potential stressor to inhibit plant growth, development and survival (Soriano *et al*., 2018; Liu *et al*., 2018a). Of course, plant tolerance to UV-B irradiation commonly depends on various factors including plant species, UV-B exposure doses and UV-B duration (Gao *et al*., 2019a; Gao *et al*., 2019b). Plant root systems have been involved in the aerial parts perception to a variety of light factors, such as red/far red light, blue light, invisible light and UV light (Feng *et al*., 2003; Ghosh & Xu, 2014; Yokawa & Baluška, 2014). Moreover, five classes of photoreceptors, including phytochromes, cryptochromes, phototropins, members of the zeitlupe family and UVR8 receptor, have been found to exist in all tissues of leaf, stem and root systems in higher plants (Chen *et al*., 2004; Rizzini *et al*., 2011; Cloix *et al*., 2012; Zhao *et al*., 2016; Bernula *et al*., 2017). These evidences have implied that the studies on the plant root responses to UV-B irradiation is of great significance in elucidating the mechanism of plant UV-B signal transduction and its responsive pathways (Wu *et al*., 2012; Yokawa and Baluška, 2014; Briggs, 2014; Yin *et al*., 2015; Liu *et al*., 2018b).

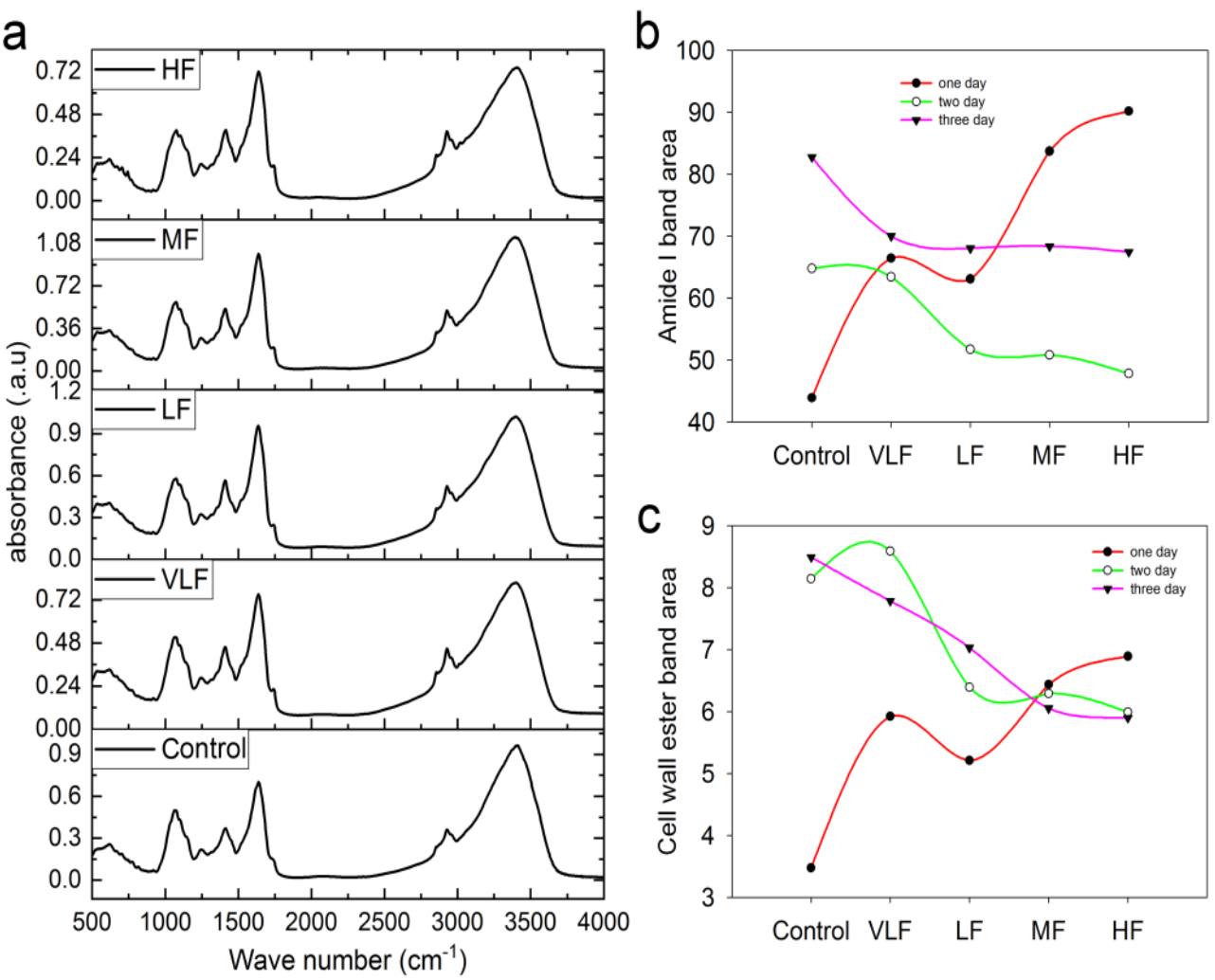

Fig. 6. FTIR absorption spectra of plant radicles.
a. FTIR absorption spectra of plant radicles; b. Amide I band area changes of plant radicles; c. Cell wall ester band area changes of plant radicles.

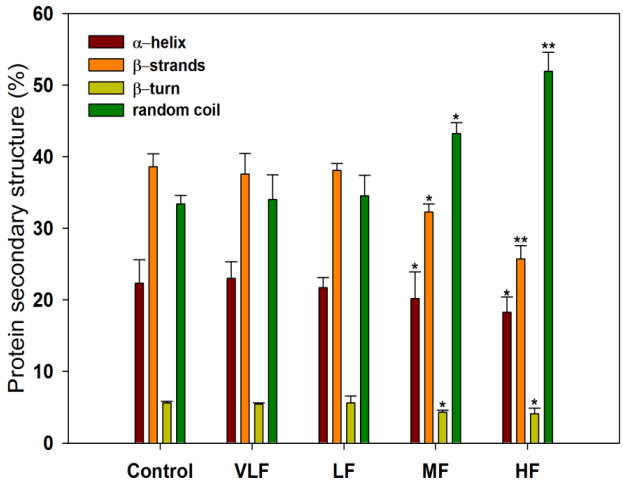

Fig. 7. The relative percentage of protein secondary structure in leaves from different intensities of UV-B.
*or ** means significant differences. * means P<0.05, ** means P<0.01.

Our experimental data suggested that different intensities of UV-B irradiation caused different degree of inhibitory effects on plant roots at multiple of morphologic, physiological and cellular levels. These changes are very similar to the biological responses of the aerial parts of the plants to UV-B irradiation (Xie *et al*., 2015; Gao *et al*., 2019b). But we have found that young roots are less resistant to UV-B irradiation than the aboveground parts in a plant. Under the lower intensities UV-B irradiation (1.0-5.0KJ m^{-2} d^{-1}) condition, alfalfa root tissues would be remarkably damaged, such as elongation inhibition, biomass reduction, and ROS-induced oxidative stress aggravation. Moreover, these damage effects will become more and more significant following the intensity of UV-B irradiation increment and UV-B exposure duration. According to the above experimental data, we have found that the inhibitory effects are the most remarkable in young roots after being irradiated for 3days by UV-B irradiation. Therefore, we further have tested root microstructure and cell vitality with radicles treated for 3d with UV-B. The results have demonstrated UV-B irradiation caused abnormal microstructure in root tissues compared to controls under normal conditions. UV-B irradiation have altered the arrangement of xylem ridge, and led to the shedding of cortical cells. Furthermore, the damage of radicle microstructure and physiological metabolisms became more significant due to the increment of UV-B. These results were consistent with previous studies (Gao *et al*., 2019b). Our experiments on alfalfa seedlings have shown that the inhibitory effects would become more serious due to the UV-B irradiation enhancement. The data of Xie *et al*., have also strongly supported our findings.

Recent works reported that the action mechanism of intracellular ROS and cell wall pectin in plant root cells might play a key role in root responses to UV-B irradiation (Yokawa & Baluška, 2014). Therefore, to further elucidate this hypothesis, we have determined proteins and cell wall ester contents and their physiochemical properties by FTIR spectroscopy method in plant root cells under five levels of UV-B irradiation conditions. The results displayed that under VLF levels of UV-B, their contents have increased to defense the adverse effects of UV-B irradiation. Whereas, under other levels of UV-B, protein and cell wall ester contents have significantly decreased. And their FTIR absorption spectra have slightly movements, and protein secondary structure abundance also have significant changes, suggesting their physicochemical properties have been changed by UV-B irradiation. However, its regulation mechanisms between cell wall esters and ROS formation still need further be explored in the next experiments.

In conclusion, the higher intensities of UV-B irradiation could cause more damages and intracellular ROS toxicities to alfalfa radicles comparison to the lower of ones. These detrimental biological effects are commonly related with the inhibitory biosynthesis of proteins and cell wall esters and their physiochemical properties alteration.

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