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FOURIER TRANSFORM INFRARED SPECTOMETRY STUDY ON EARLY STAGE OF CADMIUM STRESS IN CLOVER LEAVES

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Abstract

A technique based on Fourier transform infrared spectrometry (FTIR) was developed to detect the changes of chemical composition in the cadmium-stressed clover leaves. The obtained IR spectra were further processed by de-convolution and curve fitting for quantitatively examine the chemical contents and structure changing. Within 1h of 15mg/L Cd stress, the changes of structure and content of compounds, such as proteins, lipids and cell wall pectin synthesis, was more remarkable in clover leaves. After 24 hours of Cd stress, except saccharide, all the compounds were restored to near the control level. Our experiment suggested that the FTIR technique is applicable on study of plant stress responding.

Introduction

Following the development of metal-working industries and the extensive use of heavy metal- containing chemical fertilizer, the problem of heavy metal pollution on the environment has emerged and attracted more and more attention of people. Cadmium (Cd), a heavy metal element, is highly toxic and directly or indirectly inhibits physiological processes such as respiration, photosynthesis, cell division, plant-water relationships, N metabolism and mineral nutrition, resulting in poor growth and low biomass when at higher concentration (Farooqi *et al.,* 2009; Kabia *et al.,* 2008; Liu *et al*., 2004; Sanità & Gabbrielli, 1999; Shah *et al.,* 2008).

Cd could cause oxidative damage by affecting activity of anti-oxidative enzymes, such as superoxide dismutases (SOD), peroxidase (POD) and catalases (CAT), control the plant cellular concentrations of ROS, thus affecting the physiological activities and functions of plants (Gallego *et al.*, 1996; Cho & Park, 1997; Chaoui *et al.*, 2000; Siedlecka *et al.*, 1997). On the other hand, plants can accumulate Cd and reduce Cd stress through synthesis of phytochelatins (PCs), glutathione (GSH) or metallothioneins (MTs) (Cobbett, 2000; Lee *et al.*, 2003). Therefore, the effort to improve plant tolerance to Cd stress has been a focus of interest. However, in the process of plant growth, how Cd stress impacting on the synthesis of various metabolites, such as carbohydrate, protein and lipid, is not clearly understood yet, and the increasing knowledge will facilitate the breeding or selecting Cd resistance plant varieties.

Fourier transform infrared spectrometry (FTIR) can be used to identify the structure of unknown composition or its chemical group, and the intensity of the absorption spectra associated with molecular composition or content of the chemical group (Surewicz *et al.*, 1993; McCann *et al.,* 1992). By acquiring IR spectra from plant samples, it could detect the minor changes of macromolecule compounds, such as carbohydrate, protein, lipid, and cell wall pectin (Surewicz *et al.*, 1993; McCann *et al.,* 1992). At present, particularly in plant physiology research, FTIR has been used to identify the concrete structure of certain plant secondary metabolites (Yang & Yen, 2002; Stehfest *et al.*, 2005; Ivanova & Singh, 2003). But, on study of plants stress responding to Cd, FTIR is still a novel method.

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In this report, we conducted a comprehensive FTIR analysis of carbohydrates, proteins, lipids, and cell wall pectin from Cd-stressed clover leaves. De-convolution and curve-fitting analysis of IR spectrum could acquire accurate data, thus helping for quantitatively analyzing some functional groups. The effect of Cd on the secondary metabolites and the value of FTIR method in this field were also considerate in this study.

Materials and Methods

Growth conditions and Cd treatments: The clover were germinated directly in soil and were maintained in a growth chamber under a 16h light/8h dark, 25^{o}C cycle. After maturation, the plants were selected for uniformity and transferred to the hydroponics system. Cd was added in the form of $CdCl_2$ and the plants were grown in the presence of Cd for 1h and 24h. In each experiment, five plants were used per treatment. The hydroponics system contained 15 mg L^{-1} $CdCl_2$ and 1/5×Hoagland: KH_2PO_4, 136 mg L^{-1}; KNO_3, 101 mg L^{-1}; $Ca(NO_3)_2$, 236 mg L^{-1}; $MgSO_4$, 246. mg L^{-1}; H_3BO_3, 2.86 mg L^{-1}; $MnCl_2$•$4H_2O$, 1.81 mg L^{-1}; $ZnSO_4$•$7H_2O$; 0.22 mg L^{-1}; $CuSO_4$•$5H_2O$, 0.08 mg L^{-1}; H_2MoO_4•H_2O, 0.02 mg L^{-1}; EDTA, 7.45 mg L^{-1}; $FeSO_4$•$7H_2O$, 5.57 mg L^{-1}.

IR spectroscopy: The leaves (approximately 3-4cm^2) were taken from different plants and were pooled as one sample. Then the samples were immediately dried in an oven for 2d at 60^{o}C. Tablets for FTIR spectroscopy were prepared in an agate mortars, by mixing leaves powder (2 mg) with KBr (1:100 p/p). The absorbance spectra were measured between 400 and 4000 cm^{-1}. At least three leaves were collected and at least three spectra were obtained from each sample.

A FTIR spectrometer (FTIR-NEXUS 670, Thermo Nicolet Corporation, America) was used to collect spectra. Spectra were obtained in 32 scans co-added, 4000 resolution, and 2.0 gains. The parameters for the Fourier self-deconvolution were a smoothing factor of 15.0 and a width factor of 30.0 cm^{-1}. De-convolved and second-derivative spectra were calculated for Fourier self-deconvolution and the bands were selected and normalized to unity with Omnic 7 software. Curve-fitting of the original spectra was performed with Origin 7 software. The band position of functional groups was monitored with Knowitall 7.8 software (http://www.knowitall.com). The spectral region between 3000 and 2800 cm^{-1} was selected to analyze lipids. The spectral region between 1800 and 1500 cm^{-1} was selected to analyze proteins. The spectral region between 1200 and 1000 cm^{-1} was selected to analyze carbohydrates.

Results and Discussion

IR Monitors the changes in leaves under Cd stress: In this work, FTIR spectroscopy was used to analysis of changes in carbohydrate, protein, and cell wall. The IR absorption spectrum between 4000 and 1000 cm^{-1} was revealed on the cloverleaves IR spectroscopy (Fig. 1). In mid-IR region (2000-1000cm^{-1}) appeared large numbers of sharp peaks, indicating that the leaves have a rich chemical composition, such as carbohydrates, proteins and lipids. However, this region yielded broad and overlapped bands.

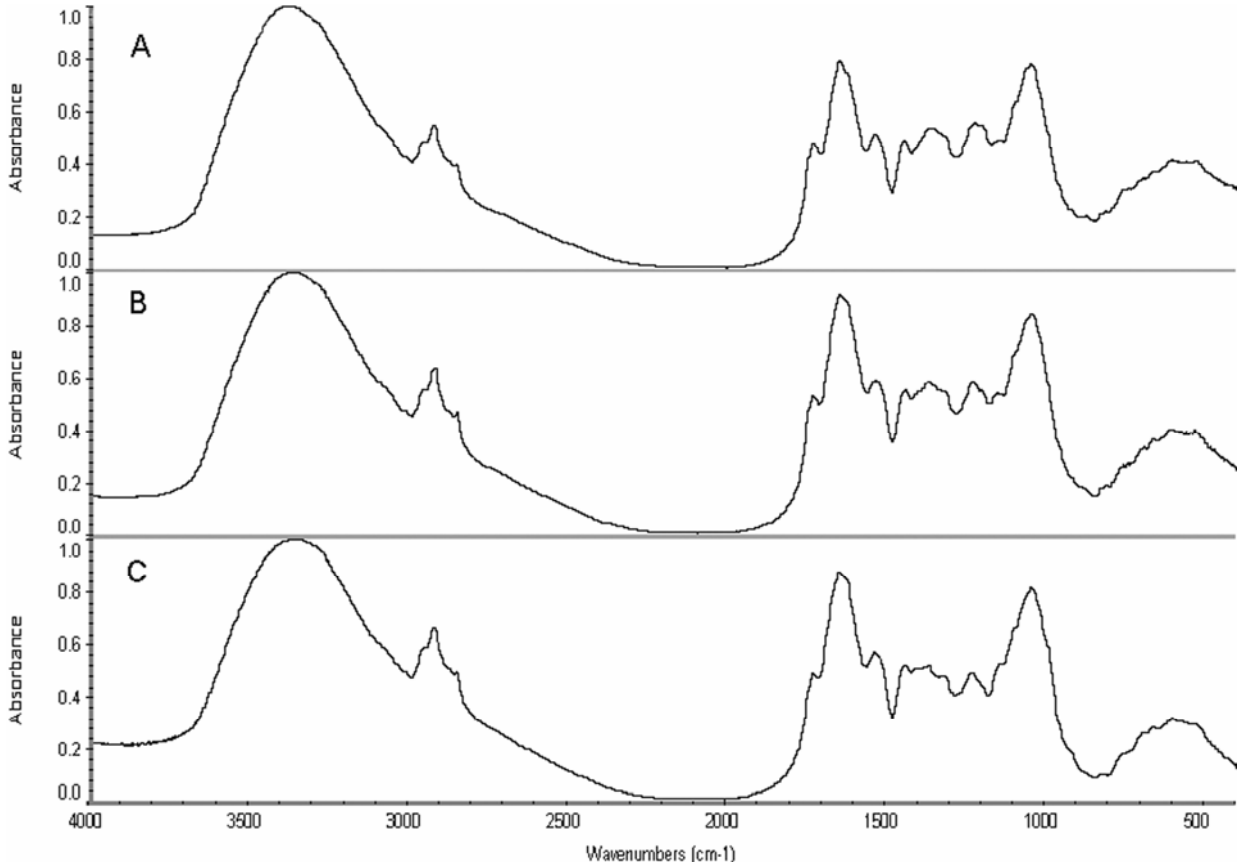

Fig. 1. FTIR absorption spectra in the 4000-1000 cm^{-1} region. (A) non-Cd treatment. (B) 15 mg/L Cd treatment for 1h. (C) 15 mg/L Cd treatment for 24h.Only one representative spectrum of each sample is shown.

Knowitall software was used to find the function groups for preliminarily analyzing IR spectra collected. The bands around 3370 cm^{-1} represent O-H and N-H stretching vibrations that are mainly generated by proteins and carbohydrates (Wolkers *et al.*, 1998). The bands between 3000 and 2800 cm^{-1} represent C-H stretching vibrations that are mainly generated by lipids (Wolkers *et al.*, 1995). The proteins absorption bands mainly located between 1800 and 1500 cm^{-1} contained amide-I and amide-II bands (Surewicz *et al.*, 1993; Stehfest *et al.*, 2005), but overlapped with other absorption bands within this region. The bands between 1500 and 1000 cm^{-1} were in the "fingerprint" region (Pan *et al.*, 2000). Amide III, the function group of nucleic acid and carbohydrates contributed to these absorption bands in the leaves.

Absorption spectrums were further processed with Fourier self-deconvolution. After this process (Fig. 2), the weak feature bands buried in some overlapped band can be enhanced, and band positions corresponding to protein, lipid, carbohydrate and cell wall pectin were clearly distinguished. The changes in protein under Cd stress: Amide-I and amide-II bands are particularly useful for determining the protein IR absorption changes. Amide-I region (1700-1600 cm^{-1}) mainly represent C=O stretching vibrations of polypeptide, which can detect changes of the overall protein conformation and content (Surewicz *et al.*, 1993). After de-convolution and curve fitting process (Fig. 2C, Table 2), three bands composed in amide-I region between 1700 and 1600 cm^{-1} were distinguished, and these bands can give additional information about the protein structure: the band around 1685 cm^{-1} assigned to the turn structure, the band around 1656 cm^{-1} assigned to the α-helix structure, and the band around 1621 cm^{-1} assigned to the β-sheet structure. To quantitatively analyze the three bands, the Gaussian peak shape was used to fit the

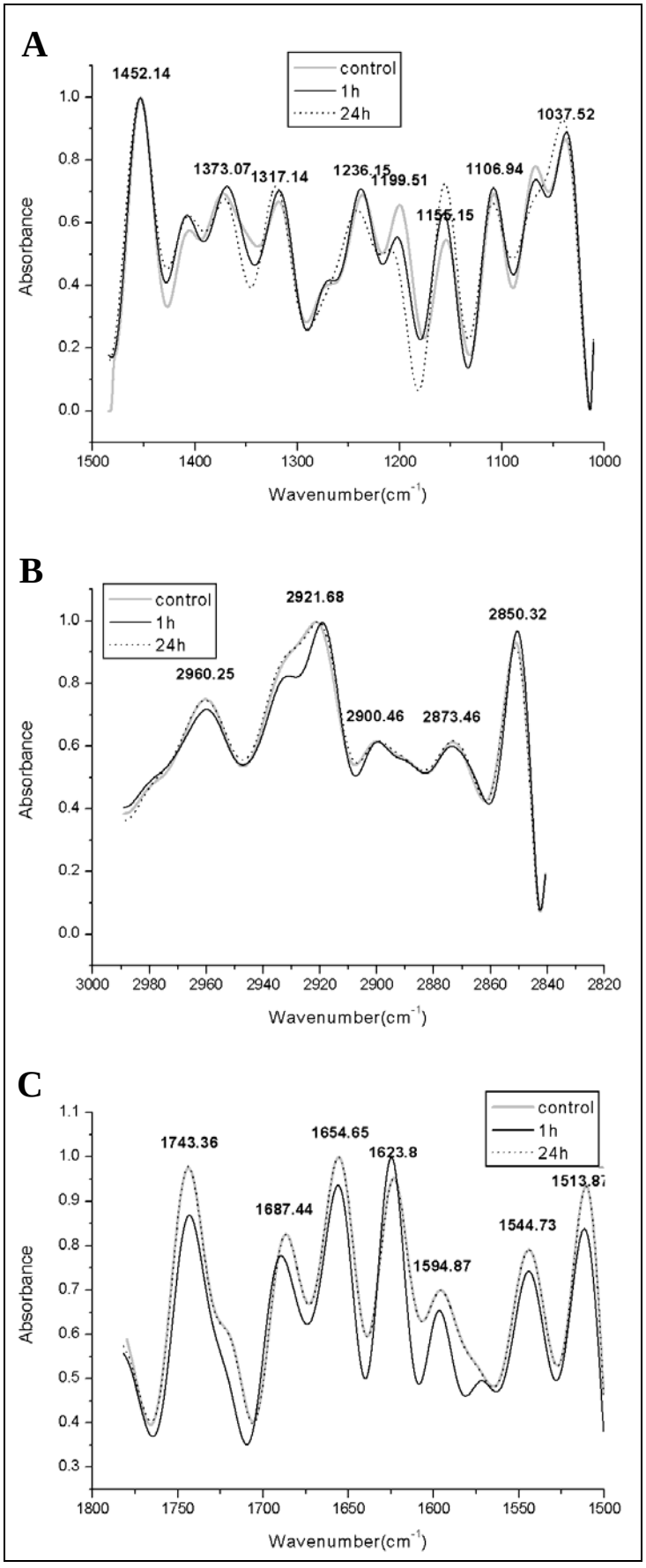

Fig. 2. Deconvolved absorption FTIR spectra in 3000-2800 cm^{-1} (A), 1800-1500 cm^{-1} (B) and 1500-1000 cm^{-1} (C) region, respectively. The A-C show the feature bands buried in overlapped bands. Only one representative spectrum of each sample is shown.

spectrum collected, and a typical curve-fitting result is shown in Table 1. After 1h of stress, the relative band areas of the turn and the β-sheet structure compared with the control have changed. The relative band area of turn structure was decreasing and the absorption strength was lower; but the corresponding value of β-sheet structure was increased and the absorption strength was enhanced. In addition, the curve-fitting results showed the amide I total band area decreased 23% which meaning the total protein contents declining remarkable, indicated that protein synthesis is sensitive to Cd. After 24h of stress, three bands returned to the control level, and the amide I total band area only decreased 1.2%. This implies that protein synthesis was mostly recovered. Similar Cd-stressed changes were also observed in the amide II band located at around 1544cm^{-1} (Fig. 2C).

The results showed that the protein synthesis is sensitive to Cd stress in clover leaves at the earlier stage, and the stability of protein secondary structures were able to changes after 1h of Cd treatment. But it was not enough to breach protein synthesis pathway and the injury was not irreversible with 15 mg/L Cd stress for 24h, and this restore phenomena maybe largely due to physiological adjust.

FTIR spectroscopy studies of protein structure are increasingly widespread used. In *Arabidopsis*, the IR spectra demonstrated salt stress can inflect the protein structure, and protein became less ordered, but following prolonged stress, this response did not persist, and protein refolded slowly (Yang & Yen, 2002). In clover leaves, changes were similar: protein returned to pre-stress level in 24h, while β-sheet, turn, and α-helix structure were inflected with Cd stress for 1h.

The Changes in lipids under Cd stress: The IR spectrum between 3000 and 2800 cm^{-1} mainly occur from lipids. De-convolution was used to enhance the resolution of IR spectra, then showed five bands uncovered (Fig. 2B), which located at approximately 2960 cm^{-1}, 2921 cm^{-1}, 2900 cm^{-1}, 2873 cm^{-1} and 2850 cm^{-1}, respectively. The bands around 2850 cm^{-1} and 2921 cm^{-1} represents C-H asym- or sym- stretching vibration, which belongs to the -CH_2 group of lipids, and the bands around 2873 cm^{-1} and 2960 cm^{-1} also represent C-H asym- or sym- stretching vibration, but it belongs to the -CH_3 group of lipid. The IR spectra were curving fitted (Table 2). After 1h of Cd stress, compared with the control, the absorption strength around 2850 cm^{-1} was enhanced and the bandwidth was decreased, whereas the absorption strength around 2921 cm^{-1} was lower and the bandwidth was increased; around 2873 cm^{-1} and 2960 cm^{-1}, the bands whose absorption strength were lower and bandwidth were increased. On the other hand, in Fig. 2B, at 2935 cm^{-1}, de-convolution enhances the resolution of small band buried in the original spectra, and it belongs to -OCH_2-group stretching vibration (http://www.chem.uni-potsdam.de), this group represents peroxides and hydroperoxides and could be considered as biomarker for indicating to peroxidate of lipid. The emerging of this peak and addition of variety of other absorption peak located in this region reflected the relative declining of -CH_2, indicated that the oxidative stress of lipid was severe after Cd exposure (Fig. 2A). After 24h of stress all bands returned to the control level and the small band at 2935 cm^{-1} disappeared. Through curve fitting analysis, in 24h the total band area between 2980 and 2845 cm^{-1} returned to the control level, and the band at 2935 cm^{-1} also disappeared. In 24h of stress, the fitting results show the total band areas (3000-2800 cm^{-1}) were similar to control. This implies that lipid have not accumulated in the clover leaf tissue. About the FTIR spectroscopy studies of plant, there was few report on -OCH_2-group stretching vibration. This provides more direct evidence for lipid peroxidation damage of Cd stress on plants.

Table 1. Analysis of the amide-I region of protein.

	A maximum (cm^{-1})	Relative area	Protein structure assignment
Control	1685	38	Turn
	1656	38	α-helical
	1621	24	β-sheet
24h	1685	38	Turn
	1656	38	α-helical
	1621	24	β-sheet
1h	1687	35	Turn
	1657	38	α-helical
	1624	27	β-sheet

Table 2. Analysis of the amide I, 3000 and 2800 cm^{-1}, 1200 and 1000 cm^{-1}, 1743 cm^{-1} region.

Absorption band region (cm^{-1})	Total band area			System
	Control	1h	24h	
Amide-I	30.79	23.63	30.39	Proteins
3000 and 2800	97.24	96.93	97.35	Lipids
1200 and 1000	114.74	115.08	92.40	Carbohydrates
1743	16.49	14.37	15.95	Pectins

IR Monitor the changes in carbohydrate under Cd stress: The IR spectra between 1200 and 1000 cm^{-1} mainly occur from carbohydrates. De-convolution showed four bands represented C-O-C stretching vibration (Fig. 2A), located at approximately 1199 cm^{-1}, 1155 cm^{-1}, 1106 cm^{-1} and 1037 cm^{-1}, respectively. After 1h of Cd stress, the total band areas between 1200 and 1000 cm^{-1} only increased 0.29% compared with the control by curve fitting analysis (Table 2), implying the carbohydrate did not accumulate obviously. But all bands strength was enhanced, indicating the carbohydrate structure has changed. After 24h of stress, the total band areas decreased 19%, and the bands at 1199 cm^{-1} and 1060 cm^{-1} lowered gradually, indicated the carbohydrate synthesis decreased and the structure kept changing, showing Cd stress changed the structure of carbohydrate in the clover leaf in 24h. Taken together, the above results means carbohydrate synthesis is sensitive to Cd stress in the clover leaves in 24h.

Literature data showed Cd stress can stimulate the metabolism of carbohydrate in the apricot leaves (Elloumi *et al.*, 2007), this indicated the carbohydrate synthesis pathway or some carbohydrate may play a critical role in the anti-oxidative response to Cd. Compared with Elloumi's results, a significant difference was exiting on carbohydrate changing profile, inflecting the dissimilar Cd resistance ability which arising from different capacity of metabolism adjustment to Cd stress.

IR monitor the changes in cell wall pectin under Cd stress: The band around 1743 cm^{-1} represents -COOR stretching vibration (Fig. 2B), which belongs to characteristic group of cell wall pectin. After 1h of Cd stress, the total band areas at 1743 cm^{-1} decreased by 12% compared with the control by curve fitting analysis (Table 2), and the band intensity was lowered, indicating the pectin synthesis decreased. After 24h of stress, this band just had a little change compared with the control, indicating the pectin synthesis returned to the control level.

However, it is regrettable that we did not detect clear characteristics spectra of nucleic acid and the influence of Cd on nucleic acid will be analyzed in the subsequent experiment by combinational using infrared microscope and FTIR device. The absorption bands of phosphodiester group were buried with other absorption bands, so, to argue whether there are produces banding with Cd, clear spectra of phosphodiester group are necessary.

At present research, the significant recovery phenomena was observed in 24h treated sample, we speculated that this due to plant native physiological adjustment mechanism. It is well known that plants can alleviate the stress of heavy metals by accumulating heavy metals, in other words, heavy metals are present as no activity or non-toxic form in plants, i.e. combined with cell wall, ion active transport into the vacuole, banding organic acids or protein (Baker, 1987). Because of Cd banding with certain plants products, the amount of Cd retained in active site can be small, then Cd toxicity is alleviated (Cobbett, 2000; Lee *et al.*, 2003). In this research, only the macroscopical and simultaneous data on biological macromolecular was obtained, and the in-depth analysis of Cd-responding second compositions need be conducted with mathematical model construction based native FTIT data.

It is very likely that Cd tolerance mechanisms may differ depending on the species (Zhou *et al*., 2008; Shah *et al.*, 2008), and this difference would be demonstrated by FTIR technique to further understand the responding and adjustment mechanism to Cd in heavy metal hyper-accumulation and sensitive plant species, ultimately accelerating plant breeding process.

In conclusion, we demonstrated Cd stress influenced carbohydrate, protein, lipid, and cell wall pectin synthesis pathway in clover leaf in 1 or 24h, however, only carbohydrate kept changing, and the metabolic balance of protein, lipid, cell wall pectin just changed in 1h. This results illuminate that Cd can affect secondary metabolism and some compounds structure have been changed.

Results obtained in this experiment suggested that FTIR was able to detect the chemical changes in Cd-stressed plants at early stages, and various spectral data can be used to analyze changes of various compounds in the plants. FTIR is more quick and convenient than other techniques for detecting physiological indicators. Due to a few amount sample needed, the entire process of plants growth can be determined with this method. Maybe the FTIR will be used extensively in the research on plants physiology due to its virtues of simple and efficient on manipulation.

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