J. Phytol. Res. **22**(2) : 271-276, 2009

MORPHOLOGICAL AND BIOCHEMICAL ALTERATIONS IN DIFFERENT CULTIVARS OF *CHEILANTHES (C. RUFA, C. TENUIFOLIA AND C. FARINOSA)* TOWARDS UV-B RAYS

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Ferms are subject of interest for many morphological and bichemical studies, in laboratories. Three species of *Cheilanthes* were chosen for present study. UV radiations at very low doses were found to show various deleterious effects. Germination per cent was found to be different in three *Cheilanthes* species. Growth parameters measured in terms of total protein contents showed the maximum amount in *Cheilanthes farinosa* (45 µg/ml) while per cent reduction in protein contents was 15.56 per cent at the 60 minutes of dose of UV-B radiations. Total amount of chlorophyll was affected by UV-B exposure in all three species of *Cheilanthes.* Maximum reduction was observed in *C. rufa* followed by *C. tenuifolia and C. farinosa.* Antioxidants were measured as total ascorbate and proline contents. *C. farinosa* showed the maximum contents of ascorbate and proline, The *C. farinosa* showed the maximum antioxidant potential in comparison with *C. rufa and C. tenuifolia* against UV-B stress.

Keywords : Ascorbate; *Cheilanthes* sps.; Free radicals; Proline; Pigments.

Introduction

Several anthropogenic human activities have led to the release of hydrocarbons and many trace gases in the atmosphere resulting in the depletion of ozone layer. Such depletion is expected to increase the amount of solar UV-B (280-320nm), which can damage the biological ecosystems. Numerous studies have been conducted on the effects of enhanced UV radiations on photosynthetic enzymes, pigments[1], proteins, seed patterns and antioxidant compound contents in plants. Hence, we were interested to see the effect of this irradiation on different *Cheilanthes* species to see their proneness to the UV-B stress. The presence/absence and increasing/decreasing property of antioxidant compounds, production of free radicals and the amount of lipid peroxidation in terms of total MDA contents, might provide significant clues to assess and evaluate the antioxidant potential of various *Cheilanthes* species against environmental stresses[2]. Plants need to have special mechanisms for adjusting the changed environment. During photosynthesis, plants harvest solar energy and assimilate it into carbon compounds which provide cellular energy and carbon skeleton for various metabolic processes. Furthermore, many groups of stresses like heavy metals, ultraviolet radiations etc. are shown to generate singlet oxygen and other active oxygen species at various sites of photosynthetic electron transport chain[3] and affect the growth of plant.

Proline is an important antioxidant that helps to maintain the osmotic potential in plant cells. It is thought that accumulated proline under environmental stress does not inhibit biochemical reactins and plays a role as an osmoprotectant during stress[4]. In addition, several possible roles have been attributed to supra-optimal levels of proline; osmoregulations under drought and salinity conditions, stabilization of proteins, prevention of heat denaturation of enzymes and conservation of nitrogen and energy for a post-stress period[5]. It is suggested that the low osmotic potential due to UV-radiations may cause proline accumulation in tissues[6].

Vitamin C or ascorbic acid is water soluble and an important antioxidant. It readily oxidizes to dehydroascorbic acid. An enzyme required for the biosynthesis of the vitamin via glucoronic acid pathway. Vitamin C reduces oxidative DNA damage and genetic mutation. It can act as a co-antioxidant by regenerating α-tocopherol from the α–tocopheroxyl radical produced during scavenging of free radicals. In addition it can reduce carcinogenesis through the stimulation of immune system.

Lipid peroxidation is a well established mechanism of cellular injury, in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to

form a complex series of compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA), upon decomposition. Lipid peroxidation will in turn result in the elevated production of free radicals[7]. Oxidative stress in cells and tissues results from the increased generation of reactive oxygen species and/or from decreases in antioxidant defense potential[8]. Lipid peroxidation can be affected by various conditions and substances such as pesticides, UV-radiations and other stresses.

Many studies have been done with emphasis on morphological, biochemical and genetic characteristics[9] of *Cheilanthes* species, with respect to ultraviolet radiations, gamma radiations and varous light qualities like red light, blue light etc. The spores of several species of ferns have a light requirement for germination[10,11].

Considering the importance of *Cheilanthes* in the ecosystem and their capacity to protect itself under stress conditions, the authors have set forth the objective of investigating the growth pattern, antioxidant system, pigment content and protein profiles of three species of *Cheilanthes (C. rufa, C. tenuifolia and C. farinosa),* grown at different exposures of UV-B radiations. These estimations could play important role in the assessment of adverse impact of stresses on *Cheilanthes* sps. growting in different habitats.

Material and Methods

Organisms and culture conditions : Spores of *C. rufa, C. tenuifolia and C. farinosa* were collected from plants growing in the kushmi forest of Gorakhpur (a tarai area of north India). The spores stored in desiccators were surface sterilized with 2% sodium hypochlorite solution and then sown uniformly on 25 ml of autoclave sterilized (15 1b/ in^2) inorganic medium at pH 5.4 in petri dishes[12]. Sowing of spores was done in an inoculation chamber fitted with germicidal UV lamp (USA). Then the plates were maintained at 24±2°C under continuous white fluorescent illumination at the intensity of 250-300 ft. c, (2700 lux).

Spores for UV treatment were placed in liquid nutrient media for 72 hours and then subjected to irradiation at 87 ergs/mm^2/sec intensity by a 15 watt Philips germicidal lamp having a peak output of 253 nm at a distance of 8 inch from the UV source. In the spore suspension an iron pin sealed in a fine capillary tube was placed on a magnetic stirrer to obtain uniform irradiation of spores. One ml of spore suspension was sucked by sterilized pipettes after every 10 minutes and dropped on agar plates. Thus, irradiation of spores for 20, 40 and 60 minutes was attained. The whole process was done in yellow light to avoid any photo reactivation. One set of UV - treated plates was kept in dark for 24 hrs and then exposed in white light while another set was exposed immediately. Protonemal length and cell number were recorded after 2 weeks. Another treatment was given after 1 month to young plants, with the same exposure time of UV-B radiations (20, 40 and 60 minutes). Then the pigments, proteins, proline and ascorbate estimations were made. Each experiment was conducted in triplicates.

Growth and photosynthetic pigments measurement : Growth determination was done by protein estimation after 10 days of inoculation. Protein contents were determined using Folins-Lowry method with lysozyme as the standard[13]. Total amount of chlorophyll from each sample of plant leaves were extracted in 80% acetone and the contents of pigments were determined by taking absorbance at 663 nm using UV-Vis spectrophotometer (Ultrospec-4000, USA).

Proline estimation : Proline contents in leaf homogenate of UV-B treated and untreated cultures were estimated according to the method of Bates *et al.*[14]. The amount of proline was calculated by comparing with standard curve. Amount of proline is represented in terms of $\mu g\ g^{-1}$ FW.

Ascorbic acid estimation : The method used is developed by Snow and Zilva[15]. The principle of this methods is based on oxidation of ascorbic acid to dehydrated ascorbic acid by shaking it with acid washed NORIT* in the presence of acetic acid. After coupling with 2, 4-Dinitrophenyl hydrazine, the solution is treated with sulfuric acid to produce the red color whose absorbance was measured at 540 nm.

**Acid washed NORIT preparation :* 200 gram NORIT (charcoal) is suspended in 1000 ml of 10% HCI, heated upto boiling point and filtered under suction. The cake is removed and stirred with 1000 ml water and filtered. This procedure is repeatd until the washing give a negative test for Fe^{3+} ions. The NORIT is then dried overnight at 110-120°C.

Results and Discussion

The spores of *C. farinosa* germinated after four days of sowing with 98% germinability, whereas those of *C. tenuifolia* and *C. rufa* germinated after 5-6 days with 95% and 93% germinability, respectively. The rate of cell division and elongation decreased with the increase of the dose. The average protonemal length of 20th day of germination in control cultures were 635.19 µm, 648.25 and 637.85 µm, in *C. rufa, C. tenuifolia* and *C. farinosa,* respectively. Among the irradiated cultures, values were getting reduced with increase in duration of doses (Fig. 1). Thus, reduction in the length of protonema was dependent on the dose of irradiation. Impairment of cell

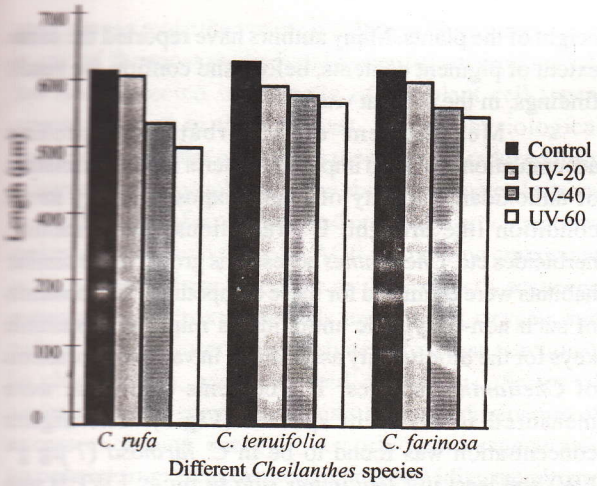

Fig.1. Effect of UV-B radiations on the length of protonema after 3 weeks of treatments.

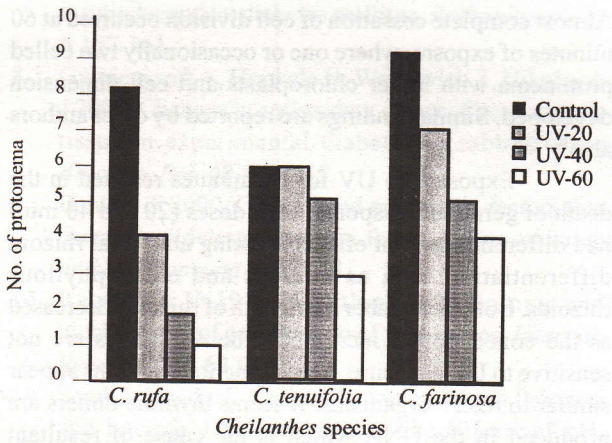

Fig.2. Effect of UV-B radiations on number of protonema after 3 weeks of treatments.

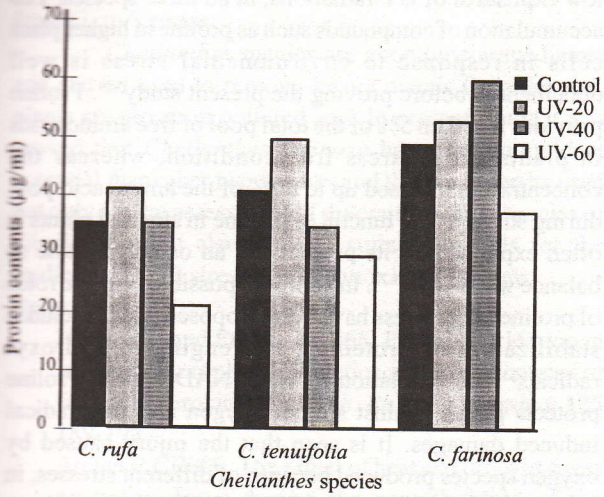

Fig.3. Effect of UV-B on protein contents
* Estimations made after 10 days of treatments

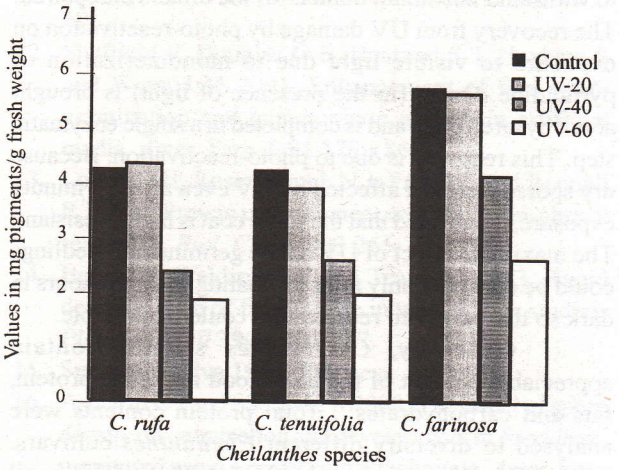

Fig.4. Effect of UV-B radiations on total chlorophyll contents in *Cheilanthes* species. (* after 10 days).

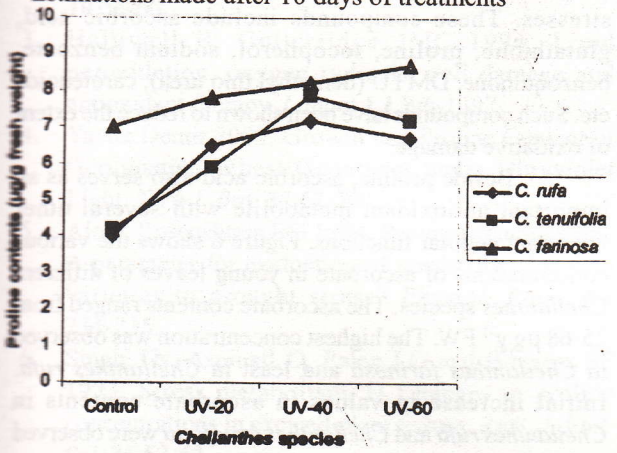

Fig.5. Effect of UV-B on Proline contents in *Cheilanthes* species

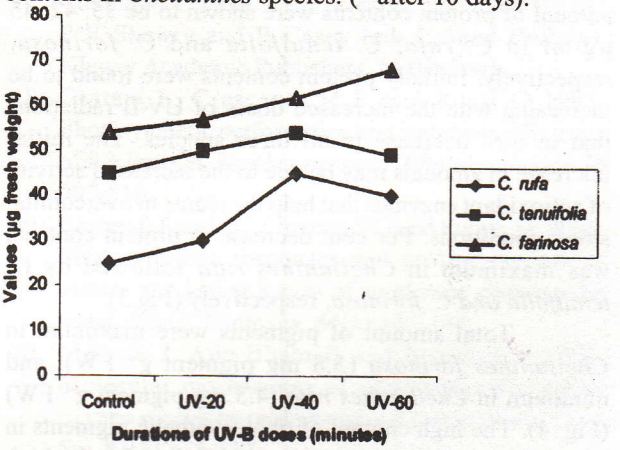

Fig.6. Effect of UV-B radiations on Ascorbate contents in *Cheilanthes* species

division occurs due to mitotic arrest at higher doses. Almost complete cessation of cell division occurred at 60 minutes of exposre, where one or occasionally two celled protonema with larger chloroplasts and cell dimension developed. Similar findings are reported by other authors also[10,12,16].

Exposure to UV for 60 minutes resulted in the death of germinating spores. Low doses (20 and 40 min) had different apparent effects showing abnormal rhizoid differentiation such as bulbous and chlorophyllous rhizoids. Both the number and length of rhizoids decreased as the concentration increased. The dry spores are not sensitive to UV exposure. The UV actions on ferns appear similar to micro-organisms. It seems thymine dimers are produced in the DNA which is the cause of resultant abnormalities. UV sensitivity of an organism depends on its ability to repair lesions caused by thymine dimers and to withstand maximum number of the dimers unrepaired. The recovery from UV damage by photo-reactivation on exposure to visible light due to monomerization of pyrimidine dimers (in the presence of light) is brought about by an enzyme and is completed in a single enzymatic step. This recovery is due to photo-reactivation. Because dry spores were not affected by UV even after 60 minute exposure, it indicated that the spore coat is highly resistant. The maximum effect of UV on the germinating seedlings could be observed only after incubating them for hours in dark so that no photo reactivation could be possible.

Generally, *Cheilanthes* species contain appreciable amount of the basic food nutrients; protein, fats and carbohydrates[17]. Total protein contents were analysed to diversify different *Cheilanthes* cultivars. Protein contents were found to be notably variable in all three species of *Cheilanthes.* In control cutures, the total amount of protein contents were shown to be 35, 42, 45 µg/ml in *C. rufa, C. tenuifolia* and *C. forinosa,* respectively. Initially protein contents were found to be increasing with the increased doses of UV-B radiations that in turn decrease in all three species. The initial inicrease in amounts may be due to the increased activity of antioxidant enzymes that help the plants in overcoming stress conditions. Per cent decrease in protein contents was maximum in *Cheilanthes rufa* followed by *C. tenuifolia and C. forinosa,* respectively (Fig.3).

Total amount of pigments were maximum in *Cheilanthes farinosa* (5.8 mg pigment g^{-1} FW), and minimum in *Cheilanthes rufa* (4.3 mg pigment g^{-1} FW) (Fig. 4). The high content of photosynthetic pigments in *Cheilanthes farinosa* might be responsible for the high growth in this plant, thus, increasing the fresh mass and height of the plants. Many authors have reported the same extent of pigment contents, before and confirm the result findings, in the present work[18].

Measurement of Ascorbate and proline accumulation is also an important criteria for determination of antioxidant capacity of plants exposed to any stress condition like drought, UV-radiations, heavy metals, herbicides etc. *Cheilanthes* accessions grown in the similar habitats were estimated for these compounds. The contents of such non-enzymatic antioxidants might give suitable keys for the biodiversity assessment in various accessions of *Cheilanthes* species. The contents of proline were measured in different species (Fig.5). The higher concentration was found to be in *C. farinosa* (7 µg g^{-1} FW) and least in *Cheilanthes rufa* (4 µg g^{-1} FW). It was observed that proline was being accumulated in leaves at low exposures of UV radiations, in all three species. The accumulation of compounds such as proline in higher plant cells in response to environmental stress is well documented before proving the present study[5,19]. Proline provides less than 5% of the total pool of free amino acids in plants under stress free condition, whereas the concentration incrased up to 80% of the amino acid pool during stress[20]. The functin of proline in stressed plants is often explained by its property as an osmolyte, able to balance water sress[6,21]. In addition, possible positive roles of proline under stress have been proposed which includes stabilization of proteins[22], scavenging of hydroxyl radicals[23] and regulation of NAD/NADH ratio. Proline protects plants against singlet oxygen and free radical induced damages. It is seen that the injury caused by oxygen species produced because of different stresses, in plants, might be suppressed by supplying antioxidants exogenously during exposure of plants to various physical stresses. These compounds include ascorbic acid, glutathione, proline, tocopherol, sodium benzoate, benzoquinone, DMTU (demethyl thio urea), carotenoids etc. Such compounds have been shown to reduce the extent of oxidative damage.

Beside proline, ascorbic acid also serves as an important antioxidant metabolite with several other important cellular functions. Figure 6 shows the various concentrations of ascorbate in young leaves of different *Cheilanthes* species. The ascorbate contents ranged from 25-68 µg g^{-1} FW. The highest concentration was observed in *Cheilanthes farinosa* and least in *Cheilanthes rufa.* Initial increase in values in ascorbate contents in *Cheilanthes rufa* and *Cheilanthes tenuifolia* were observed with the increase in UV-exposure. Later on the values were found to be decreasing at high doses. Though the values

were always more than control cultures. The ascorbic acid is one of the most studied and powerful antioxidant[24]. It has been detected in majority of the plant cell types, organelles and in the apoplast. Under physiological conditions, ascorbic acid exists mostly in the reduced form in leaves and chloroplasts[23]. The ability to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid the main AOS detoxifying compound in the aqueous phase. Ascorbic asid can directly scaverge superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water *via* ascorbate peroxidase reaction[25]. In chloroplasts, ascorbic acid acts as a cofactor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy[23]. Ascorbic acid regenerates tocopherol from tocopheroxyl radical providing membrane protection. In addition, ascorbic acid has been implicated in the regulation of the cell division, cell cycle progression from G_1 to S phase.

Cheilanthes species are good phytoremediators and can be used to remove heavy metals from the soil, plants are grown in polluted soil. In present study, it was proved that *Cheilanthes farinosa* has more antioxidant potential than other two species and this study can be used not only for the assessment of diversity in these species of *Cheilanthes* but also provide suitable models for the studies on UV-B stresses and antioxidant systems.

References

1. Heath R L and Packer L 1968, Photoperoxidation in isolated chloroplast I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **125** 189-198.
2. Archer R C 2000, The effect of heat and drought stress on native ferns during the growth cycle of the following year. *Hardy Fern Foundation Quarterly* **10** 56-60.
3. Halliwell B, Gutterridge JMC 1994, Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. *Lancet* **1** 1396-1397.
4. Yavuz Demir 2000, Growth and Proline Content of Germinating Wheat Genotypes under Ultraviolet Light. *Turk J. Bot.* **24** 67-70.
5. Aloni, Rosenshtein BG 1984, Proline accumulation : A parameter for evaluation of sensitivity of tomato varieties to drought stress? *Physiol. Plant.* **61** 231-235.
6. Singh TN, Aspinall D, Paleg LG and Boggess SF 1973, Stress metabolism. II Changes in proline concentrations in excised plant tissues. *Aust. J. Biol. Sci.* **26** 57-63.
7. Levy U, Zaltzber H, Ben Amotz A, Kanter Y, Aviram M 1999, α-Carotene affects antioxidant status in non-insulindependent diabetes mellitus. *Pathophysiology* **6** 157-161.
8. Gumieniczek A, Hopkala H, Wojtowich Z, Nikolajuk J 2002, Changes in antioxidant status of heart muscle tissue in experimental diabetes in rabbits. *Acta Biochim. Pol.* **49** 529-535.
9. Banks J A 2000, Genetic and epigenetic factors that control sex determination in ferns. *J. Reproduction and Development* **46** 41-42.
10. Raghavan V 1980, Cytology, physiology and biochemistry of germination of fern spores. *Internat. Rev. of Cytol.* **62** 69-118.
11. Sarah L Nondorf, Melissa A Dooley, Maria Palmieri and Lucinda J Swatzell 2003, The Effects of pH, Temperature, Light Intensity, Light Quality and Moisture Levels of Spore Germination in *Cheilanthes feei* of Southeast Missouri. *Amer. Fern J.* **93**(2) 56-69.
12. Sheffield E, Douglas G E, Hearned S J, Huxham S and Wynn J M 2001, Enhancement of fern spore germination and gametophyte growth in artificial media. *Amer. Fern J.* **91** 179-186.
13. Lowry O H, Rosenbrough N J, Farr A L and Randall R J 1951, Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193** 265-275.
14. Bates LS, Waldren R P and Teare ID 1973, Rapid determination of free proline in water-stress studies. *Plant and Soil* **39**(1) 205-207.
15. Snow and Zilva 1943, *Biochem. J.* **37** 630.
16. Ashcroft C J and Sheffield E 2000, The effect of spore density on germination and development in *Pteridium,* monitored using a novel cultue technique. *Amer. Fern J. 90* 91-99.
17. Cuming A C 1999, LEA proteins. pp. 753-780, In : P R Shewry and R Casey (eds.). *Seed Proteins.* Kluwer Academic Publishers, Netherlands.
18. Gratani L, Crescente M F and Rossi G 1998, Photosynthetic performance and water use efficiency of the fern *Cheilanthes persica. Photosynthetica* **35** 507-516.
19. Cechin I, Rossi SC, Oliveira VC and Fumis TF 2006, Photosynthetic responses and proline content of mature and young leaves of sunflower plants under water deficit. *Photosyn.* **44**(1) 143-146.
20. Matysik J, Alia Bhalu B and Mohanty P 2002, Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. *Curr. Sci.* **82**(5) 525-532.
21. Delauney AJ and Verma DPS 1993, Proline

biosynthesis and osmoregulation in plants. *Plant J.* **4** 215-223.

22. Anjum F, Rishi V and Ahmed F 2000, Compatibility of osmolytes with Gibbs energy of stabilization of proteins. *Biochim. Biophys. Acta* **1476** 75-84.
23. Smirnoff N 2000, Ascorbic acid: metabolism and function of a multifaceted molecule. *Curr. Op. Plant Biol.* **3** 229-235.
24. Horemans N, Foyer CH and Asard H 2000, Transport and action of ascorbate in the plant plasma membrane. *Trends Plant Sci.* **5** 263-267.
25. Noctor G and Foyer CH 1998, Ascorbate and glutathione : keeping active oxygen under control. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49** 249-279.