BIOLOGIA PLANTARUM 37 (3): 355-362, 1995

Growth, $^{14}CO_2$ fixation, activites of photosystems, ribulose 1,5-bisphosphate carboxylase and nitrate reductase in trees as affected by simulated acid rain

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Abstract

In seedlings of the tropical tree species *Erythrina variegata* Lam. and *Hardwickia binata* Roxb. exposed to different acidic mist (H_2SO_4, pH 5, 3 and 2) for 5 d significant reduction in seedling growth, biomass accumulation and $^{14}CO_2$ fixation were determined. In isolated chloroplasts a decrease in the activities of photosystem 2 and whole electron transport chain was observed only at pH 3 and 2, but no significant change in photosystem 1 activity was observed. SDS-PAGE analysis of crude leaf extracts of ribulose 1,5-bisphosphate carboxylase (RuBPC) indicated a significant loss of 55 and 15 kDa polypeptides at pH 2 in *Erythrina*. The reduction in the RuBPC activity in seedlings grown under acidic mists correlated well with CO_2 fixation.

Key words: *Erythrina variegata*, *Hardwickia binata*, H_2SO_4, polypeptides, photosynthesis.

Introduction

Wet deposition by mist, rain, hail, sleet or snow is often collectively called acid precipitation or acid rain (when pH is less than 5.6). They bring sulphates and nitrates to soil systems, increase soil acidity and cause mobilization or leaching of nutrient cations which then threaten soil fertility. Acid rain damages leaves due to local acidity which causes weathering or degradation of the waxy leaf cuticle. Strong acids oxidize and hydrolyse the waxy esters and release some of the long fatty acid chains from the waxy matrix. This changes the water repelling (hydrophobic) characteristics of leaf cuticle and increases the wettability (Wellburn 1988,

Received 1 August 1994, *accepted* 17 October 1994.

Acknowledgements: This work was supported by a financial grant from the Society for Social Forestry Research and Development, Tamilnadu, India to K.M.C. We thank Prof. T.M. Haridasan for helpful comments and suggestions. We also thank Mr.S. Balakrishnan for carefull typing of the manuscript.

Scherbatskoy 1989). Recent studies have been made on physiological and biochemical responses of crop plants to acidic mists (Muthuchelian *et al.* 1993, 1994) and on the effects of gaseous air pollutants on higher plants (Weigel *et al.* 1989).

The degree of injury by acid rain depends to a large extent on the effective dosage which is a function of both the concentration and the period of exposure (Wellburn 1988). Acid rain affects chlorophyll content (Takemoto *et al.* 1987, Franzen *et al.* 1989, Smith *et al.* 1990, Sasek *et al.* 1991, Muthuchelian *et al.* 1994), stomatal conductance (Chappelka *et al.* 1988, Saxe 1991, Muthuchelian *et al.* 1994) and net photosynthetic rate (Takemoto *et al.* 1987, Martens *et al.* 1989, Muthuchelian *et al.* 1994). Acid rain affects also certain enzyme activities like nitrate reductase and RuBPC (Muthuchelian *et al.* 1993), or glutathione reductase and ascorbate peroxidase (Chen *et al.* 1991).

Studies on growth, photosynthesis and other physiological as well as biochemical changes due to simulated acidic mists in tropical tree seedlings are rare. Hence, investigations were carried out to understand the influence of simulated acidic mist on growth and photosynthetic acitivities in *Erythirna variegata* Lam. and *Hardwickia binata* Roxb.

Materials and methods

Plants: Pre-soaked seeds *Erythrina variegata* Lam. and *Hardwickia binata* Roxb. were germinated in earthen pots in a greenhouse (temperature 28 ± 2 °C; relative humidity 65 ± 5 %; maximum irradiance 1800 μmol(PAR) m^{-2} s^{-1}; photoperiod 14 h). After 15 d, when the cotyledonary leaves had expanded, the seedlings were given the appropriate acidic mist treatment. Seedlings (five per pot) were exposed to three different pH treatments (pH 5, 3 and 2) with H_2SO_4 solution acidic mists and deionized water as control according to Muthuchelian *et al.* (1993). Seedlings were sprayed daily, until the leaves were completely wet (09.30) for 5 d with a sprayer and gave 2 mm precipitation. The soil was covered with a plastic hood to avoid indirect effects of acid precipitation. Both control and treated pots were arranged in a randomised complete block design with five replication per treatment. Fully expanded leaves of plants were harvested 40 d after emergence for determining the growth attributes (Radford 1967).

Chlorophyll (Chl), carotenoids and soluble protein contents were determined spectrophotometrically by the methods of Arnon (1949), Goodwin (1954) and Lowry *et al.* (1951), respectively. The content of total carotenoids in the 80 % acetone extract was determined using an absorbance coefficient of A_{473} 1 % = 2500.

Total soluble sugars and starch: Sugars were thoroughly extracted with boiling 80 % ethanol and estimated by the anthrone reagent method (Dubois *et al.* 1956). Soluble starch was extracted and its concentration was determined following the method of Mc Cready *et al.* (1950).

Activities of electron transport: Type II broken chloroplasts were prepared from control and acid mist treated seedlings of *E. variegata* and *H. binata* according to the method of Reeves and Hall (1973). Photosynthetic partial reaction mediated by PS2 and PS1 were measured as described by Noorudeen and Kulandaivelu (1982). Whole chain electron transport ($H_2O \rightarrow MV$) was measured as described by Armond *et al.* (1978).

$^{14}CO_2$ fixation was measured on leaf segments in 5 cm^3 of 50 mM KH_2PO_4-KOH buffer (pH 7.5) containing 50 mM $MgCl_2$, 35 mM NaCl, and 10 mM $NaHCO_3$ irradiated for 5 min (40 W 'white' fluorescent tubes). $NaH^{14}CO_3$ (1850 kBq) was injected into the reaction medium and incubated at 25 °C for 30 min under white light (900 μmol m^{-2} s^{-1}). The reaction was stopped by cold acetic acid (final concentration 10 %). The leaf segments were washed and ground in an inocubation medium and the volume was made upto 3 cm^3. Aliquots of 10 mm^3 of the homogenate were loaded on to the *Whatman No.1* filter paper discs and dried at room temperature under incandescent lamps. The radioactive carbon fixed was measured using the *Packard model 2425* liquid scintillation counter.

Extracts and assay of RuBPC activity: Fully expanded leaves were cut into small pieces and homogenized in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM dithiothereitol or 10 mM 2-mercaptoethanol, and 0.25 mM EDTA. The extract was clarified by centrifugation at 20 000 *g* for 10 min. The clear supernatant was decanted slowly and used as the crude RuBPC source. All these steps modified method of Bowes and Ogren (1972). The incubation mixture contained 50 mM DTT and 10 mM $NaH^{14}CO_3$ (9.25 kBq μmol^{-1}) in a total volume of 2.0 cm^3. The reaction mixture was placed in *Pyrex* tubes. After flushing with N_2 for 3 min, the tubes were sealed with serum caps and gently shaken in a water bath at 32 °C for 3 min. Aliquots of 0.2 cm^3 of the enzyme extract were then injected through the serum cap into the mixture to initiate the reaction. After 3 min at 32 °C the reaction was stopped by injecting 0.2 cm^3 of 6 M glacial acetic acid. The known aliquots were transferred to *Whatman No. 3* filter paper discs, dried under infra-red lamp, and the radioactivity was determined using a *Packard model 2425* liquid scintillation counter. Soluble proteins were estimated by the procedure of Lowry *et al.* (1951).

SDS-PAGE was performed as described by Laemmli (1970) using a polyacrylamide gradient of 8 - 16 % gel.

Nitrate reductase (NR) activity: The *in vivo* NR activity was assayed according to Jaworski (1971) with suitable modifications. Leaf segments of 1 cm^2 (0.25 g) were incubated in 5 cm^3 of incubation medium composed of 100 mM KH_2PO_4-KOH (pH 7.5), 100 mM KNO_3, and 1 % (v/v) *n*-propanol until the tissue was completely wet. Incubation was carried out in the dark at room temperature (27 $\pm$ 2 °C) for 60 min. Suitable aliquots of the infiltration medium were then assayed for nitrite with sulfanilamide and N-(1-naphthyl) ethylene diamine dihydrochloride (Muthuchelian *et al.* 1990).

Results and discussion

Acidic mists (pH 3 and 2) greatly retarded the shoot elongation and leaf expansion in both plant species (Table 1). Besides the overall stunted growth of the plant, leaves were small, thin and leathery in texture with less cuticular waxes on the upper surface of leaves. Morphological symptoms such as curling of leaves, deterioration of the cuticular barriers and cracking of the thin waxy plugs which cover the stomata were also observed. Similar reduction in leaf size and cracking of the thin waxy plugs have been reported in tree seedlings exposed to acidic mists (Wellburn 1988, Van Elsacker and Impens 1989). Moreover, acidic mist significantly reduced leaf area, leaf area index, specific leaf mass and relative growth rate in comparison with control seedlings (Table 1).

Table 1. Effect of acidic mist on plant height, leaf area index, relative growth rate, specific leaf mass and plant biomass in *Erythrina* and *Hardwickia* seedlings. The seedlings were sprayed with solutions of H_2SO_4 (pH 5, 3, 2) and deionized water (control). Figures in parentheses are percent inhibition with reference to respective control. Values are the means of 25 seedlings.

Parameter	Plant	Control	pH 5		pH 3		pH 2	
Plant height	*Erythrina*	77.00	65.00	(16)	49	(36)	29.00	(62)
[cm]	*Hardwickia*	45.00	40.00	(11)	36	(22)	29.00	(36)
Leaf area index	*Erythrina*	0.0084	0.0077	(8)	0.0062	(26)	0.0051	(39)
	Hardwickia	0.0034	0.0033	(3)	0.0028	(22)	0.0025	(31)
Relative growth rate	*Erythrina*	2.78	2.45	(12)	1.6	(42)	0.98	(65)
[g kg^{-1} s^{-1}]	*Hardwickia*	1.34	1.28	(5)	1.0	(25)	0.85	(37)
Specific leaf area	*Erythrina*	0.85	0.75	(12)	0.37	(56)	0.23	(73)
[kg m^{-2}]	*Hardwickia*	0.51	0.48	(6)	0.39	(24)	0.28	(45)
Plant biomass	*Erythrina*	12.9	10.9	(16)	6.4	(50)	3.1	(76)
[g(d.m.) $plant^{-1}$]	*Hardwickia*	4.3	3.9	(9)	3.2	(26)	2.3	(47)

Acidic mist treated seedlings had lower biomass accumulation than the control seedlings. Conspicuous reductions in biomass of 16, 50, 76 % in *Erythrina* and 9, 26 and 47 % in *Hardwickia* were observed at pH 5, 3 and 2, respectively. This was in agreement with the findings of Van Elsacker and Impens 1988, Van Elsacker *et al.* 1989, and Lee *et al.* (1990).

Seedlings exposed to acidic mists had lower contents of Chl *a+b* and carotenoids (Table 2), that could be due to an inhibition in biosynthesis or an increase in breakdown of pigments or their precursors.

Total leaf soluble protein content was reduced drastically by 64 % in *Erythrina* and by 46 % in *Hardwickia* at pH 2 (Table 2). The relatively low level of soluble protein might have been due to decrease in the synthesis of RuBPC, the major soluble protein of leaf. A loss of leaf protein would partially account for damaged chloroplasts or be the result of inhibition of protein synthesis (Allen *et al.* 1978, Muthuchelian *et al.* 1993).

The reduction in starch and sugar contents were resemblance with each other in both the tree seedlings. At pH 2, the decrease in starch and sugar contents was higher in *Erythrina* than *Hardwickia*. The reduction in RuBPC activity of seedlings grown under acidic mist correlated well with the $^{14}CO_2$ fixation. $^{14}CO_2$ fixation of *Erythrina* and *Hardwickia* was affected at pH 2. Inhibitions of $^{14}CO_2$ fixation by 17, 37, 68 % in *Erythrina* and 9, 17, 24 % in *Hardwickia* at pH 5, 3 and 2, respectively, were observed. Reduction in $^{14}CO_2$ fixation of seedlings exposed to acidic pH 2 was an indirect effect, due to the inhibition or destruction of photosynthetic pigments or due to the increased stomatal diffusive resistance (Martens *et al.* 1989, Muthuchelian *et al.* 1994).

Table 2. Effect of acidic mist treatment on total chlorophyll (Chl), carotenoids, protein content, starch and sugar contents, $^{14}CO_2$ fixation, RuBPC and nitrate reductase (NR) activities of *Erythrina* and *Hardwickia* seedlings. The seedlings were sprayed with H_2SO_4 (pH 5, 3, 2) and deionized water (control). Figures in parentheses are percent inhibition with reference to respective control. Values are the means of six determinations from each replicate pot in various treatments and controls.

Parameter	Plant	Control	pH 5		pH 3		pH 2	
Chl *a+b*	*Erythrina*	2.77	2.35	(15)	1.75	(37)	1.58	(43)
[g kg^{-1}(f.m.)]	*Hardwickia*	1.32	1.15	(13)	1.03	(22)	0.92	(20)
Leaf soluble proteins	*Erythrina*	23.00	19.25	(14)	9.05	(60)	8.0	(64)
[g kg^{-1}(f.m.)]	*Hardwickia*	11.00	10.20	(7)	8.05	(22)	5.9	(46)
Starch	*Erythrina*	22.78	20.15	(12)	16.70	(27)	13.36	(41)
[g kg^{-1}(f.m.)]	*Hardwickia*	19.05	17.38	(9)	14.36	(25)	12.61	(38)
Sugar	*Erythrina*	17.76	16.65	(6)	12.65	(29)	11.98	(33)
[g kg^{-1}(f.m.)]	*Erythrina*	16.09	16.00	(5)	13.31	(21)	12.81	(24)
$^{14}CO_2$ fixation	*Hardwickia*	59.00	49.00	(17)	37.00	(37)	20.00	(68)
[μmol(CO_2) kg^{-1}(Chl) s^{-1}]	*Erythrina*	46.00	42.00	(9)	38.00	(17)	35.00	(24)
RuBPC activity	*Hardwickia*	70.00	65.00	(7)	47.00	(33)	35.00	(54)
[μmol(CO_2) kg^{-1}(prot.) s^{-1}]	*Hardwickia*	58.00	55.00	(5)	45.00	(23)	41.00	(30)
NR activity	*Erythrina*	124.00	120.00	(17)	50.00	(60)	7.00	(94)
[μmol(NO_2) kg^{-1}(f.m.) s^{-1}]	*Hardwickia*	2.65	2.51	(9)	1.68	(37)	0.94	(65)

Marked changes in the RuBPC activity in leaf extracts were observed in both tree seedlings when expressed on protein basis. The inhibition of RuBPC activity increased with an increase in the pH of the acidic mist in both the tree seedlings; it may be due to protein destruction and/or enzyme inactivation.

RuBPC peptide profiles of crude leaf extract of *Erythrina* seedlings (Fig. 1) showed that acidic mist (pH 2) markedly affected both the large (55 kDa) and small (15 kDa) subunits. 55 and 15 kDa polypeptides was marginally decreased at pH 5 and 3, respectively.

The overall photosynthetic electron transport ($H_2O \rightarrow MV$) showed a significant inhibition of 8, 20 and 60 % in *Erythrina* and of 5,12 and 40 % in *Hardwickia* at pH 5, 3 and 2, respectively (Table 3). PS1 activity showed no significant change in

acidic mist treated seedlings. In contrast to this, PS 2 activity mediated by BQ declined significantly, more in *Erythrina* than *Hardwickia* at pH 2. No clear conclusion has been reached on the mechanism(s) or site(s) of inhibition of electron transport systems by acidic mist treatments. Destruction of structural integrity of chloroplast thylakoids induced by acidic mist treatment contributes to the decrease in PS 2 activity, but does not affect much the PS 1 activity (Table 3).

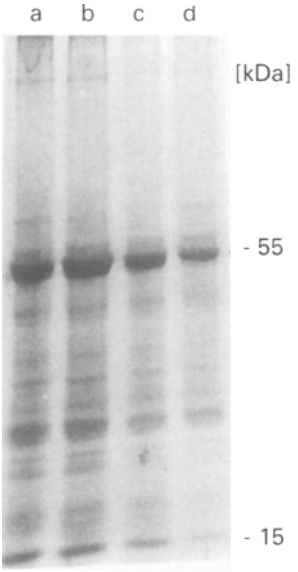

Fig. 1. SDS-PAGE profiles of crude leaf ribulose 1,5-bisphosphate carboxylase proteins in *Erythrina* seedlings: control (*a*) and acidic mists treatments, pH 5 (*b*), 3 (*c*) and 2 (*d*).

Table 3. Effect of acidic mist treatment on the whole chain and photosystems (PS) 2 and 1 electron transport in *Erythrina* and *Hardwickia* chloroplasts. The seedlings were sprayed with H_2SO_4 (pH 5, 3, 2) and deionized water (control). Figures in parentheses are percent inhibition with reference to respective controls. Values are the means of six determinations from each replicate to in various treatments and controls.

Parameter	Plant	Control	pH 5		pH 3		pH 2	
Whole chain, $H_2O \rightarrow MV$	*Erythrina*	76	70	(8)	61	(20)	30	(60)
[$\mu mol(O_2)\ mg^{-1}(Chl)\ s^{-1}$]	*Hardwickia*	54	52	(4)	47.5	(12)	32.4	(40)
PS 2, $H_2O \rightarrow BQ$	*Erythrina*	87	78	(10)	71	(18)	32	(63)
[$\mu mol(O_2)\ mg^{-1}(Chl)\ s^{-1}$]	*Hardwickia*	75	72	(4)	67.5	(10)	43.5	(42)
PS 1, $DCPIPH_2 \rightarrow MV$	*Erythrina*	115	110	(4)	108.1	(6)	105.8	(8)
[$\mu mol(O_2)\ mg^{-1}(Chl)\ s^{-1}$]	*Hardwickia*	120	118	(1)	116.4	(3)	112.8	(6)

NR activity was more reduced at pH 2 than at pH 5 and 3 in both the tree species (Table 2). The changes in the intercellular pH levels due to acidic mists treatment

(Wellburn 1988) may affect transfer of nitrate (substrate) from vacuolar storage pool to active cytoplasmic pool accessible to the enzyme. The inhibition of NR activity may also be due to an inhibition of protein synthesis (Muthuchelian *et al.* 1993) or it may have stemmed out from decreased photosynthetic supply in the acidic mist treated seedlings. A good correlation was observed between the decrease in NR activity and leaf protein content in response to acidic mist.

Our experiments confirmed that *E. variegata* was more sensitive to acidic mist than *H. binata*. The hard leathery upper epidermis of *H. binata* leaf could partially resist the acidic mists. Further studies are in progress to find out the molecular mechanism(s) or site of inhibition in photosynthetic machinery by acidic mists.

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