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Protective effect of triacontanol against acidic mists in *Samanea saman* (Jacq.) Merrill seedlings: Differential responses in growth, $^{14}CO_2$ fixation, ribulose-1,5-bisphosphate carboxylase, and electron transport activities

K. MUTHUCHELIAN*, V. MEENAKSHI, and N. NEDUNCHEZHIAN

Centre for Biodiversity and Forest Studies, School of Energy Sciences, Madurai Kamaraj University,
Madurai – 625 021, India

Abstract

Seedlings of tropical leguminous tree *Samanea saman* (Jacq.) Merrill were exposed for 7 d to acidic mist (AM, induced by H_2SO_4) of pH 5.6, 4.0, and 2.0. AM significantly reduced seedling growth (root and shoot length, leaf density, leaf area, fresh and dry mass accumulation) and photosynthetic activities. In thylakoids isolated from leaves treated at pH 4.0 and 2.0 a decrease in the activities of photosystem (PS) 2 and whole chain electron transport was observed, but PS1 activity did not change. When the seedlings were subsequently sprayed with triacontanol (TRIA), the AM effect was partially or completely reversed indicating that TRIA can protect from AM effects. The artificial electron donors, diphenylcarbazide (DPC) and hydroxylamine (NH_2OH), markedly restored the loss of PS2 activity in AM (pH 2.0) treated leaves. This is the first report of alleviating the AM by TRIA in tropical tree seedlings.

Additional keywords: cow tamarind; donor side; electron transport; fresh and dry matter; leaf density and area; nitrate reductase; photosystems 1 and 2; root and shoot length.

Introduction

Photosynthetic production can be influenced by a number of environmental factors such as temperature, nutrients, water, salts, heavy metals, herbicides, radiation, and pollutants (see, *e.g.* Muthuchelian 1987, Kulandaivelu *et al.* 1989, Muthuchelian *et al.* 1989). Only recently growth and metabolic reactions of crop plants to different air pollutants (Wellburn 1988), physiological and biochemical changes within the plants (Ziegler 1986), and the effects of gaseous air pollutants on higher plants from a physiological point of view (Weigel *et al.* 1989) were studied. Wet deposition by mist, rain, hail, sleet, or snow is often collectively called precipitation or acid rain (with pH of less than 5.6). Acid rain damages leaves, causing local acidity, which then causes weathering or degradation of the waxy leaf cuticle. Strong acids oxidise 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 cuticles and increases wetability (Wellburn 1988, Scherbatskoy 1989).

Acid rain also has inhibitory effects on growth (Keith and Dochinger 1989, Lee *et al.* 1990), chlorophyll content (Takemoteo *et al.* 1987, Franzen *et al.* 1989, Smith *et al.* 1990, Sestak *et al.* 1991), stomatal conductance (Chappelka *et al.* 1988, Saxe 1991), spectral characteristics (Ashendon and Williams 1988), and net photosynthetic rate (Takemoteo *et al.* 1987, Martens *et al.* 1989, Muthuchelian *et al.* 1992). Acid rain affects several enzymes including changes in nitrite and nitrate reductase, RuBP carboxylase (Muthuchelian *et al.* 1992), and glutathione reductase and ascorbate peroxidase (Chen *et al.* 1991) activities. Studies of physiological and biochemical changes due to acidic mist (AM) have been few in tropical tree seedlings.

Triacontanol [$CH_3(CH_2)_{28}CH_2OH$] (TRIA) and its second messenger L(+)-adenosine stimulate plant growth

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*Fax: 0452-2459139, e-mail: drchelian1960@yahoo.co.in.

Abbreviations: AM – acidic mist; BQ – *p*-benzoquinone; Car – carotenoids; Chl – chlorophyll; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; MV – methyl viologen; PQ – plastoquinone; PS – photosystem; RuBPC – ribulose-1,5-bisphosphate carboxylase; TRIA – triacontanol.

(Ries 1991). Most profound effect of TRIA is increase in growth, biomass, and photosynthetic activities, and in contents of free amino acids, reducing sugars, and soluble proteins (Muthuchelian *et al.* 1995).

The objective of this study was to investigate whether TRIA can protect plants from AM by assessing the concerted effect of TRIA on acid mist treated *Samanea saman* (rain tree, "cow tamarind") seedlings with particular emphasis on growth, pigments, and ribulose-1,5-bisphosphate carboxylase (RuBPC) and photosynthetic activities. *S. saman* is widely grown in tropical environment and its wood is useful as timber, craft wood, and fuel wood. The leaves and pods are used for food and feed having high nutritive and nitrogen fixing capability.

Materials and methods

AM/TRIA treatments: Seedlings of *S. saman* (Jacq.) Merrill were grown at Centre for Biodiversity & Forest Studies, Madurai Kamaraj University, Madurai [temperature 30±2 °C, relative humidity 65±5 %, maximum irradiance (PAR) of 1 600 μmol m^{-2} s^{-1}, photoperiod 14 h]. Fourty days after sowing the seedlings (five per pot) were divided into eight equal groups: (*1*) Plants (control, pH 6.8) were maintained at soil moisture close to the field capacity throughout their growing period (control). (*2* to *4*) Plants were grown at three different concentrations of AM (pH 5.6, 4.0, and 2.0, sulphuric acid : nitric acid by volume 7 : 3) (for details see Muthuchelian *et al.* 1995). (*5*) Seedlings were sprayed by hand pump sprayer with growth stimulator TRIA [1 mg kg^{-1}(H_2O)] (*NOCIL*, India) with *Tween* 20 (0.1 %, m/v) added as a surfactant and the above concentration was standardised as in Muthuchelian *et al.* (1992, 1995). Care was taken to wet both sides of the leaf. (*6* to *8*) Seedlings were treated by AMs (pH 5.6, 4.0, and 2.0) and simultaneously were sprayed with TRIA. Both control and treated pots were arranged in a randomised complete block design with five replicates per treatments.

Growth and biochemical measurements: Fully expanded leaves of plants were harvested 50 d after emergence and growth analysis components were calculated according to Radford (1967). Contents of Chl, Car, and soluble proteins were determined spectrophotometrically by the methods of Lichtenthaler (1987) and Lowry *et al.* (1951), respectively. Total sugar was thoroughly extracted with boiling 80 % ethanol and estimated by the anthrone reagent method (Dubois *et al.* 1956). Soluble starch was extracted and its concentration determined following the method of McCready *et al.* (1950).

Activities of electron transport: Thylakoid membranes were isolated from the leaves as described by Berthhold *et al.* (1981). Whole chain electron transport ($H_2O \rightarrow$ MV) and partial reactions of photosynthetic electron transport mediated by photosystem (PS) 2 ($H_2O \rightarrow$BQ) and 1 ($DCPIPH_2 \rightarrow$MV) were measured as described by Nedunchezhian *et al.* (1997). Thylakoids were suspended at 10 g(Chl) m^{-3} in the assay medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl, and 100 mM sucrose supplemented with 500 μM BQ.

Rate of DCPIP photoreduction was determined as the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer. The reaction mixture (3 cm^3) contained 20 mM Tris-HCl, pH 7.5, 5 mm $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 100 μm DCPIP, and thylakoid membranes equivalent to 20 μg Chl. Where mentioned, the concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

Extracts and assay of RuBPC activity: Leaves were cut into small pieces and homogenised in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.25 mM EDTA. The extract was clarified by centrifugation at 10 000×*g* for 10 min. The clear supernatant was decanted slowly and used as the RuBPC. The assay of RuBPC activity was measured as described by Nedunchezhian and Kulandaivelu (1991).

$^{14}CO_2$ fixation: $^{14}CO_2$ 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 $NaHCO_3$ and irradiated for 5 min (40 W "white" fluorescent tubes). $NaH^{14}CO_3$ (1 850 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 incubation medium and the volume was made up to 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.

Nitrate reductase (NR) activity *in vivo* 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 buffer (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±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.

Results and discussion

Changes in the growth characteristics of *Samanea* seedlings exposed for 7 d with and without AM/TRIA are shown in Fig. 1. AM (pH 4.0 and 2.0) greatly retarded the root and shoot elongation. Leaf density and leaf area was also affected. Besides this overall stunting of the plant, leaves were small, thin, and leathery in texture with less cuticular waxes on their upper surfaces. Morphological symptoms, such as curling of leaves, deterioration of the cuticular barriers, and cracking of the thin waxy plugs, which cover the stomata, were observed. Similar reductions in leaf size and cracking of the thin waxy plugs were reported in tree seedlings exposed to AM (Radford 1967, Muthuchelian *et al.* 1995).

AM-treated seedlings had lower fresh and dry mass accumulation than the control seedlings. Dry mass was reduced by 14, 25, and 32 % in *S. saman* seedlings at pH of 5.6, 4.0, and 2.0, respectively (Fig. 1). The declines in fresh and dry mass accumulation observed in the AM-treated seedlings were in agreement with previous findings (Muthuchelian *et al.* 1992, 1994). TRIA treatment resulted in significantly higher root and shoot elongation, leaf density and area, and fresh and dry biomass accumulation. The results of this and previous (Muthuchelian *et al.* 1990, 1992, 1994, 1995, 1996, 2001) studies suggest that the lipophillic TRIA may act on cell membranes to produce 9-β-(+) adenosine (Ries and Wert 1992). This substance is rapidly translocated throughout the plant causing a cascade of metabolic events and resulting in increases in growth and dry matter. An explanation for increased growth and dry mass in *Samanea* may come from eliminating all possibilities except water. It is possible that water is metabolically incorporated *via* saccharide and/or fat hydrolysis (Ries and Wert 1982). There may also be an increase in hydroscopicity (physically bound water) of the TRIA-treated plants, because of the large increase in contents of free amino acids and reducing sugars, which constitute about 30 % of the dry mass of the plants. Any growth attribute to TRIA may not be simply caused by increased water uptake and cell elongation but also may be due to an increase in cell numbers (Hangarter and Ries 1978). TRIA treatment resulted in significantly greater root and shoot elongation, leaf density, leaf area, fresh and dry biomass accumulation in salt, drought, flooding, and cadmium stressed *Erythrina* seedlings (Muthuchelian *et al.* 1995, 1996, 2001). These results suggest that the above mentioned growth retardation by AM might be ameliorated by TRIA application (Muthuchelian *et al.* 1990, 1995).

Seedlings exposed to various AM treatments had a lower Chl content per unit fresh mass than the control (Table 1). This was due to the decrease in both Chl *a* and Chl *b* contents. The decrease was highly conspicuous at a mist of pH 2.0. Similar reduction was also observed in carotenoid (Car) contents. The reason for the reduction in Chl content under AM could be due to an inhibition in biosynthesis or to an increase in breakdown of pigments or their precursors (Muthuchelian *et al.* 1994). TRIA treatment resulted in significantly larger Chl and Car contents in control and in all AM treatments. Even at a mist of pH 2.0, Chl and Car were protected. The

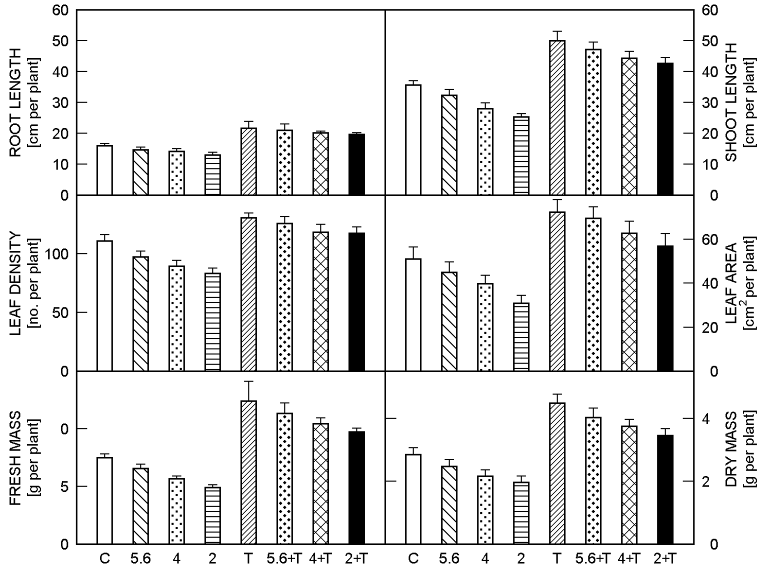

Fig. 1. Effect of TRIA (1 mg m^{-3}) and acidic mist (AM) on root, shoot length, leaf density, leaf area, and fresh and dry masses of *Samanea saman* seedlings. Seedlings were harvested after 7 d of AM/TRIA treatment for growth analysis. Means±SD, *n* = 20. C – control; AM treatments [pH]: 5.6, 4.0, 2.0; T = TRIA; 5.6+T = AM pH 5.6 + TRIA; 4+T = AM pH 4 + TRIA; 2+T = AM pH 2 + TRIA.

increased pigment contents of TRIA-treated leaves were attributed to an increase in the number and size of chloroplasts, the amount of Chl per chloroplast, and better grana development (Muthuchelian *et al.* 1990). The higher amounts of pigments in TRIA-treated leaves, even in the presence of AM, suggest that TRIA can prevent symptoms of acidity. TRIA increased the synthesis of both Chl *a*, Chl *b*, and Car in flooded, salt stressed, and cadmium stressed *E. variegata* seedlings (Muthuchelian *et al.* 1995, 1996, 2001).

The contents of starch and sugar decreased in AM-treated seedlings (Table 1). Similar observations were made in crop plants and tree species (Muthuchelian *et al.* 1994, 1995). TRIA reverses the effect. TRIA application also increases the amount of starch and sugar in salt stressed, flooded, and cadmium stressed *E. variegata* seedlings (Muthuchelian *et al.* 1994, 1996, 2001). Soluble starch and sugars may function as compatible solutes in cytosol of *Samanea* where they can significantly contribute to the adjustment to acidic stress (Muthuchelian *et al.* 1996). Content of total soluble proteins was reduced appreciably at a mist of pH 2.0 (Table 1). The seedlings exposed to high acidity had a relatively lower amount of soluble proteins, and this may be due to a decrease in the synthesis of fraction I protein (RuBPC), the major soluble protein of leaf. A loss of leaf protein would partially account for damaged chloroplasts or be the result of inhibition or protein synthesis (Muthuchelian *et al.* 1994, 1995).

The whole chain electron transport was markedly inhibited in AM-treated seedlings. However, the PS1 activity was much less diminished (Muthuchelian *et al.* 1995).

In contrast to PS1, the PS2 activity measured by both benzoquinone (BQ) and DCPIP was significantly inhibited (Fig. 2). DCPIP collects electrons after plastoquinone (PQ) but BQ at the reducing side of PQ (Lien and Bannister 1971). The PS2 activity loss due to AM action must be prior to PQ in the electron transport. To locate the possible site(s) of inhibition in the PS2 reaction, we followed the DCPIP photoreduction supported by various exogenous electron donors in thylakoids isolated from control and AM-treated leaves with or without TRIA. Wydrzynski and Govindjee (1975) have shown that $MnCl_2$, DPC, NH_2OH, and HQ could donate electrons to PS2. Fig. 3 shows the electron transport activity of PS2 in the presence and absence of three of the above compounds. Among the various electron donors, DPC and NH_2OH were more effective than $MnCl_2$ in restoring PS2 activity in AM-treated leaves. Hence, AM did induce changes on the donor side of PS2, prior to the site of donation by NH_2OH and close to the site of donation by DPC. TRIA reduced the inhibition by AM of both whole chain and PS2 activities. TRIA enhances monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) contents on a protein basis (Shripathy and Sivakumar Swamy 1994). MGDG may be involved in the protection of photosystem activities against AM. The extensive change in fatty acid composition of thylakoid

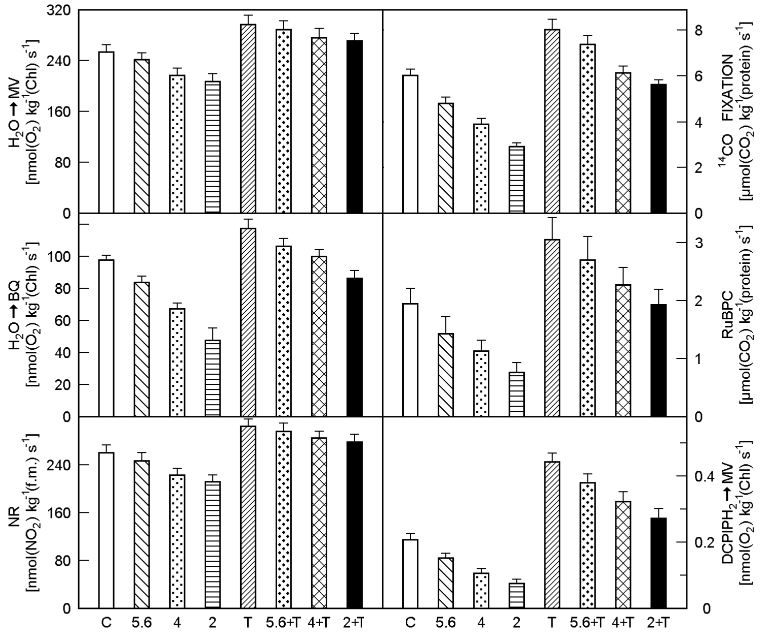

Fig. 2. Effect of TRIA (1 mg m^3) and acidic mist (AM) on whole chain ($H_2O \rightarrow MV$), PS2 ($H_2O \rightarrow BQ$), and PS1 ($DCPIPH_2 \rightarrow MV$) photosystem activities, $^{14}CO_2$ fixation, RuBPC and nitrate reductase (NR) activities in *Samanea saman* chloroplasts isolated after 7 d of AM/TRIA treatment. Means±SD, *n* = 5. C – control; AM treatments [pH]: 5.6, 4.0, 2.0; T = TRIA; 5.6+T = AM pH 5.6 + TRIA; 4+T = AM pH 4 + TRIA; 2+T = AM pH 2 + TRIA.

Table 1. Effect of different acidic mist treatments on the contents of pigments, starch, sugars, and proteins [g kg^{-1}(f.m.)] in non-treated and TRIA treated 40-d-old *Samanea saman* seedlings. Figures in parentheses are % inhibition as compared to controls. Means±S.D.; *n* = 5.

Acidic mist (pH)	Chl	Car	Starch	Sugars	Proteins
0 (control)	2.62±0.12 (0)	0.620±0.031 (0)	19.1±0.9 (0)	29.8±1.3 (0)	42.3±2.0 (0)
5.6	2.14±0.19 (18)	0.520±0.025 (16)	16.8±0.7 (12)	25.6±1.1 (14)	35.6±1.9 (16)
4.0	1.70±0.08 (35)	0.427±0.021 (31)	13.8±0.5 (28)	22.1±1.0 (26)	30.5±1.4 (28)
2.0	1.46±0.06 (44)	0.372±0.018 (40)	11.5±0.5 (40)	19.1±0.9 (36)	24.6±1.1 (42)
0 +TRIA	2.81±0.14 (0)	0.688±0.034 (0)	24.8±1.1 (0)	36.6±1.2 (0)	48.6±2.3 (0)
5.6+TRIA	2.55±0.12 (9)	0.619±0.030 (10)	23.3±1.1 (6)	33.7±1.2 (8)	44.2±2.1 (9)
4.0+TRIA	2.42±0.11 (14)	0.605±0.031 (12)	20.6±1.0 (17)	31.8±1.1 (13)	41.8±2.0 (14)
2.0+TRIA	2.19±0.09 (22)	0.543±0.026 (21)	17.9±0.8 (28)	25.6±1.0 (30)	37.9±1.5 (22)

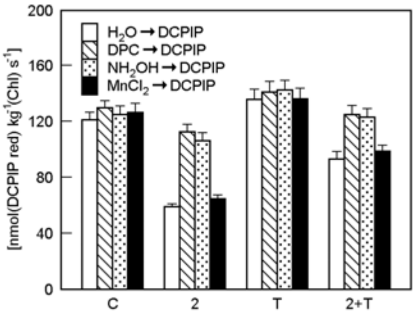

Fig. 3. Effect of various exogenous electron donors on PS2 activity ($H_2O \rightarrow DCPIP$) in control chloroplasts and 7-d acidic mist/TRIA treated chloroplasts. Means±SD, *n* = 5. C = control; 2 = AM pH 2.0; T = TRIA; 2+T = AM pH 2.0 + TRIA.

membrane correlating with electron transport activities is an essential part of processes by which cells control membrane function and stability (Muthuchelian *et al.* 1996). Significant decrease in the ratio of unsaturated to saturated fatty acids in acid treated seedlings (data not shown) perhaps reflects depletion of unsaturated fatty acids by lipid peroxidation due to AM. TRIA application rapidly increases the unsaturated fatty acid contents (oleic acid 18 : 1; linoleic 18 : 2; linolenic 18 : 3) which would help to maintain the thylakoid membrane in an appropriate state in *Erythrina* seedlings. This could be the possible mode of action by which the synthesised unsaturated fatty acids in TRIA-treated *Samanea* seedlings partially protect the cellular systems against AM. The effects on PS1 activity were much smaller in size but of a similar character (Fig. 2). Similar trend was also observed in *E. variegata* seedlings by the application of TRIA and cadmium stress (Muthuchelian *et al.* 2001). RuBPC activity was reduced markedly in AM treated leaves, whereas TRIA treatment increased it (Fig. 2). The relatively low content of soluble proteins in AM-treated seedlings might be due to decrease in the synthesis of RuBPC, the major soluble protein of leaf. A loss of proteins in AM-treated leaves would partially account for damaged chloroplasts or could be the result of inhibition of protein synthesis or proteolytic degradation of the enzyme (Swirshi and Gepstein 1985). The reduction in the overall photosynthetic rate correlates well with the decreased RuBPC activity in acid-treated leaves. A marked reduction of RuBPC activity was observed at pH 2.0 (Fig. 2). Such reduction was due to the inhibition of protein synthesis induced by AM. The reduction in RuBPC activity in AM-treated seedlings and the improvement by TRIA treatment correlated with the $^{14}CO_2$ fixation (Fig. 2). The reduction in $^{14}CO_2$ fixation of AM-treated seedlings was probably an indirect effect due to the destruction of photosynthetic pigments (as evidenced by the present results).

Seedlings grown in the presence of AM had a relatively low NR activity (Fig. 2) that was ameliorated by TRIA application. The reduction in NR activity may reflect a balance between synthesis and inactivation on one hand, and degradation or inactivation on the other. The changes in intercellular pH values induced by acidic stress might decrease the transfer of nitrate (substrate) from a storage pool to an active cytoplasmic pool accessible to the enzyme. The inhibition of NR activity might be also due to the inhibition of protein synthesis or might have stemmed out from the decreased rate of photosynthetic supply in AM-treated leaves.

The enhancement of growth by TRIA might result from an increase in effective leaf area, stimulation of photosynthesis, RuBPC and nitrate reductase activities, modification in partitioning of photosynthesis, or from their co-operative effects (Muthuchelian *et al.* 1994, 1995, 1996). In the present work, the application of TRIA to AM-treated seedlings reduced the AM effects by partially restoring/maintaining the photosynthetic machinery. In these plants increases in growth, pigments, RuBPC, NR, and photosynthetic activities were demonstrated.

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