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Technique for retardation of leaf senescence of six ericoid wild plant taxa using ascorbic acid

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ABSTRACT

Efficacy of a growth promoter ascorbic acid (Vitamin C) on senescence deferral action was analysed using leaf discs of six wild ericoid plant taxa. Changes of some biochemical parameters like chlorophyll, protein, soluble and insoluble carbohydrates, RNA and DNA as well as activity of catalase enzyme were analysed as reliable senescence indices during detached leaf senescence of these six species under dark condition. With the progress of ageing duration from zero to 144 hours the levels of chlorophyll and proteins in leaf discs gradually declined in both control and ascorbic acid treated samples. However, in the chemical / Vitamin-C treated samples the rate of decline was found to be much slower. Concomitantly the levels of insoluble carbohydrate, RNA and DNA started declining right from 48, 96 and 144 hours of observation period both in treated and untreated samples. And here also ascorbic acid arrested the rapid rate of reduction of the levels of the biochemical parameters. On the other hand, soluble carbohydrate level started increasing irrespective of the treatments during the whole observation periods. However, the magnitude of increase was found to be low in case of the leaf samples which experienced treatment with ascorbic acid. The activity of the enzyme catalase was found to decrease progressively during the four observation periods (0, 48, 96 and 144 hours) regardless of the

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treatments. Ascorbic acid partially averted the rapid fall of the enzyme activity during the ageing periods. Ascorbic acid, a nonconventional senescence deferred thus seems to be a potent senescence deferral phytohormone /chemical / vitamin at least in case of six ericoid wild plant taxa.

***Keywords*:** Leaf senescence, Ericaceae, Ascorbic acid, India

1. INTRODUCTION

Senescence is a programmed deteriorative phenomenon occurring within cells, tissues, organs and organisms, which is culminated in the death of the concerned plant part or the organisms as a whole (Leopold and Kriedemann, 1975; Pati, 2007). As the process of senescence takes place at an exceedingly faster rate under detached condition of plant parts, the effect of any chemical having influence on the regulation of senescence can be quickly determined (Sabater, 1984).

Deferral of senescence by plant hormones like cytokinins is well established (Richmond and Lang, 1957; Vanstaden *et al*., 1988; Biswas and Ghosh, 1999). However, some gibberellins and auxins are reported to defer senescence in a number of plant species, but their efficiency is mostly not at par with cytokinins (Biswas and Ghosh, 1999). Again, many plant species do not respond positively for deferment of senescence in presence of auxin types.

In the present experiment, an attempt was made to ascertain whether ascorbic acid, a member of vitamin class (Vit. C), can regulate senescence of the experimental Ericoid plant species namely *Enkianthus deflexus* (Griff.) C. K. Schneid. var. *acuminatus* Panda & Sanjappa, *Gaultheria stapfiana* Airy Shaw, *G. hookeri* C. B. Clarke, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Vaccinium glauco-album* C. B. Clarke and *V. nummularia* C. B. Clarke under detached leaf condition.

Regulation of plant senescence by any chemical agent can be expeditiously and almost accurately determined under detached condition of plant parts. In fact, deteriorative processes during senescence of detached leaves simulate grossly with that of attached leaves under natural condition, the main difference being the speed at which the processes run. Thus, the principal aim of this work was to evaluate the efficiency of ascorbic acid on senescence retardation of six wild plant taxa, which are reported to be very sensitive towards any chemicals with regard to exhibiting senescence regulatory action (Biswas and Ghosh, 1999).

2. MATERIALS AND METHODS

In this investigation the experimental plants used were six highly elevated wild angiospermic plants of the family Ericaceae such as *Enkianthus deflexus* (Griff.) C. K. Schneid. var. *acuminatus* Panda & Sanjappa, *Gaultheria stapfiana* Airy Shaw, *G. hookeri* C. B. Clarke, *G. trichophylla* Royle var. *ovata* Panda & Sanjappa, *Vaccinium glauco-album* C. B. Clarke and *V. nummularia* C. B. Clarke.

The plant taxa were first carefully surface blotted using blotting paper. Uniformly sliced leaf discs, taken from mature leaves of the plants were treated with aqueous solution of ascorbic acid (100 μg/ml) or distilled water (control) in Petri dishes containing filter paper.

The experimental set-up was kept in dark condition and thus allowed the leaf discs to experimental treatment with ascorbic acid for 144 hours. At an interval of 48 hours the filter papers were remoistened with the test chemical or distilled water. At 48 hours intervals the biochemical data recorded include: chlorophyll, protein, soluble and insoluble carbohydrates, RNA and DNA contents as well as activity of the enzyme catalase.

Chlorophyll: To determine chlorophyll level, leaf tissues (100 mg) of each treatment were taken in 5 ml methanol in test tubes and kept in dark under laboratory conditions for 48 hours. The supernatant was decanted off and leaf samples were rinsed repeatedly with a little amount of methanol. The supernatant and washing materials were pooled together to make the final volume 10 ml. Subsequently, the absorbance of the chlorophyll extracts was measured at 650 nm and total chlorophyll content was estimated following Arnon's principle (1949).

Protein: The chlorophyll free leaf samples (100 mg) were solubilized by treating the leaf tissues with 0.5M NaOH at 80 ºC for one hour after making the samples free from phenol as per the method of Kar and Mishra (1976). A definite volume was made with the extraction medium. It was then estimated by allowing the protein solution to react with Folin phenol reagent and subsequent measuring of the OD values at 650 nm as per the method of Lowry *et al*. (1951).

Soluble carbohydrate: This was analysed from 100 mg leaf samples homogenised thoroughly using 80% boiling ethanol (5 ml). The homogenates in watch glasses were then evaporated to dryness and this was followed by removal of chlorophylls using solvent ether. The sugar was then pooled in test tubes by washing the watch glass using 80% ethanol. A definite volume was made with 80% ethanol and this was the source for soluble carbohydrate.
After necessary dilution, 1 ml sample was taken in test tube and 3 ml freshly prepared, precooled anthrone reagent (0.2% in conc. H_2SO_4) was added. The intensity of green colour was measured at 610 nm. This method was followed essentially as per Mc Cready *et al.* (1950).

Insoluble carbohydrate: Insoluble carbohydrate level was estimated from the residual material after extraction of leaf tissues (100 mg) with 80% boiling ethanol. The residue was dissolved in 25% H_2SO_4 and extraction was done at 80 ºC for 30 minutes. After necessary dilution, 1 ml of the acid extracted sample was taken in test tube and 3 ml freshly prepared, precooled anthrone reagant was added and the intensity of the green colour was measured in the same way as done in case of soluble carbohydrate.

Nucleic acids: Extraction of nucleic acids (RNA and DNA) was made from 100 mg fresh leaves following the method described by Cherry (1962). The levels of RNA and DNA were estimated from a common stock employing the method of Markham (1955) modified by Choudhuri and Chatterjee (1970).

RNA: For the estimation of RNA, 3 ml of diluted nucleic acid extract in a test tube was treated with an equal volume of freshy prepared orcinol reagent (1g orcinol powder in 100 ml of conc. HC1 containing 100 mg 0.1% $FeC1_3$, $6H_2O$) and boiled in a water bath for 20 min with glass marbles at the test tube tops. The mixture was then cooled, necessarily diluted and the intensity of green colour was measured at 700 nm.

DNA: 1 ml of the nucleic acid extract in a test tube was mixed with 5 ml freshly prepared diphenyl amine reagent (100 ml glacial acetic acid, BDH, AR + 2.7 ml H_2SO_4 + 1 g AR grade diphenyl amine). The mixture was boiled in a water bath for 30 min with glass marble at the top of the test tubes. After cooling, the intensity of the colour was measured at 610 nm.

Catalase: Leaf tissues (500 mg) of each treatment were homogenised with 8 ml of chilled 0.1M phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 6.8). The homogenate was centrifuged at 3000 g for 15 min followed by 10,000 g for 20 min. The volume of the supernatant was made up to 10 ml with the same buffer, and this was assayed following the method of Snell and Snell (1971) modified by Biswas and Choudhuri (1978). The reaction mixture for catalase consisted of 1 ml of the above extract and 2 ml of H_2O_2 (0.05M), incubated together at 37 ºC for 30 min. The reaction was stopped by adding 1 ml 0.1% titanyl sulphate ($TiSO_4$) in 25% H_2SO_4 (v/v). After centrifugation for 6000 g for 10 min the intensity of yellow colour was measured at 420 nm.

3. RESULTS AND DISCUSSIONS

Chlorophyll and protein contents started declining rapidly with the advancement of leaf ageing irrespective of the treated and control samples. However, ascorbic acid arrested the rapid loss of both chlorophyll and protein levels (Table 1).

Table 1. Effect of ascorbic acid (A.A; 100 µg/ml) on the changes in chlorophyll (Chl, mg/g fresh weight) and protein (Pr, mg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Leaf discs were treated with IAA or distilled water for 144 hours and the data were recorded at 48 hour intervals.

Plant taxa	Treatments (µg/ml)	Hours after leaf ageing							
		0		48		96		144	
		Chl.	Pr.	Chl.	Pr.	Chl.	Pr.	Chl.	Pr.
1. *Enkianthus Deflexus* var. *acuminatus*	Control	1.24	2.21	1.00	1.12	0.87	1.00	0.67	0.75
	A.A.	1.24	2.21	1.12	1.72	1.01	1.24	0.93	1.04
	LSD(P=0.05)	NC	NC	0.11	0.13	0.12	0.14	0.11	0.09
2. *Gaultheria hookeri*	Control	1.32	2.37	1.00	1.32	0.75	1.12	0.62	0.62
	A.A.	1.32	2.37	1.12	1.72	1.00	1.48	0.96	0.87
	LSD(P=0.05)	NC	NC	0.11	0.21	0.57	0.28	0.14	0.18
3. *G. stapfiana*	Control	1.18	2.15	0.82	1.70	0.58	1.02	0.35	0.56
	A.A.	1.18	2.15	1.05	1.97	0.85	1.54	0.72	0.98
	LSD(P=0.05)	NC	NC	0.07	0.16	0.06	0.10	0.05	0.07

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	1.12 1.12 NC	2.12 2.12 NC	0.92 1.12 0.11	1.02 1.11 0.08	0.68 1.00 0.07	0.82 0.92 0.16	0.48 1.12 0.17	0.62 0.78 0.11
5.*Vaccinium nummularia*	Control A.A. LSD(P=0.05)	1.45 1.45 NC	2.65 2.65 NC	1.16 1.32 0.10	1.85 2.32 0.15	0.80 1.05 0.07	1.32 1.85 0.10	0.66 0.80 0.05	0.78 1.60 0.07
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	1.85 1.85 NC	2.54 2.54 NC	1.52 1.70 0.12	1.70 2.25 0.18	1.30 1.61 0.10	1.20 1.60 0.11	0.75 1.01 0.08	0.64 1.05 0.07

NC: Not calculated.

A differential result was observed when the changes of these two variables were recorded with stress-induced ageing duration. Soluble carbohydrate was found to increase while insoluble carbohydrate steadily declined with the progress of ageing duration (Table 2). Ascorbic acid ameliorated the ageing-induced rapid rise of the sugar levels as well as the progressive loss of the insoluble carbohydrate levels.

Table 2. Effect of ascorbic acid (A.A; 100 µg/ml) on the changes in soluble carbohydrate (Sol., mg/g fresh weight) and insoluble carbohydrate (Insol., mg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**							
		0		**48**		**96**		**144**	
		Sol.	**Insol.**	**Sol.**	**Insol.**	**Sol.**	**Insol.**	**Sol.**	**Insol.**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	0.90 0.90 NC	7.98 7.98 NC	1.09 0.98 0.22	6.98 7.24 1.23	2.34 1.27 0.73	5.34 6.00 1.21	3.87 2.98 1.00	4.39 5.38 1.01
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	0.87 0.87 NC	6.25 6.25 NC	1.13 0.98 0.55	5.25 6.00 1.23	2.21 1.87 1.03	4.26 5.23 1.02	3.32 2.36 1.23	3.54 4.12 1.92
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	0.85 0.85 NC	5.50 5.50 NC	1.05 0.92 0.08	4.05 4.68 0.38	1.95 1.25 0.11	3.52 4.05 0.35	2.30 1.52 0.15	2.15 2.75 0.21

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	0.98 0.98 NC	4.98 4.98 NC	1.11 1.00 0.11	3.98 4.18 0.35	1.98 1.28 0.25	2.28 3.08 1.01	2.21 1.98 1.00	1.20 2.98 1.12
5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	0.65 0.65 NC	7.82 7.82 NC	0.85 0.78 0.07	6.75 7.09 0.38	1.68 1.02 0.10	4.68 5.51 0.28	1.85 1.15 0.11	3.60 4.01 0.20
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	0.96 0.96 NC	6.75 6.75 NC	1.15 1.05 0.10	4.59 4.90 0.39	1.59 1.15 0.12	3.80 4.25 0.35	2.38 1.58 0.14	2.88 3.44 0.22

NC: Not calculated.

Both RNA and DNA (Table 3) levels gradually decreased with the stress-induced ageing of leaf samples. Ascorbic acid was found to significantly check the loss of both the nucleic acid levels and the effect was particularly significant when data were recorded after 48 hours of leaf ageing and subsequent observations.

Table 3. Effect of ascorbic acid (A.A.; 100 µg/ml) on the changes in RNA (µg/g fresh weight) and DNA (µg/g fresh weight) contents in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**							
		0		**48**		**96**		**144**	
		RNA	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	407.0 407.0 NC	74.6 74.6 NC	367.2 397.2 12.1	61.3 70.0 0.91	258.4 302.3 14.5	51.4 67.0 11.3	201.6 249.6 12.6	40.4 51.3 14.6
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	401.5 401.5 NC	78.8 78.8 NC	378.8 399.3 12.8	67.6 75.9 10.7	298.4 300.1 10.7	52.6 67.1 11.3	199.5 209.4 13.0	42.4 57.3 13.4
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	401.5 401.5 NC	55.8 55.8 NC	338.7 375.9 20.75	47.5 50.9 3.35	295.8 338.5 15.92	41.9 47.8 2.92	201.5 225.6 13.70	28.8 34.9 2.01

4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	412.1 412.1 NC	65.3 65.3 NC	398.1 401.3 24.1	54.8 60.6 22.1	298.3 305.1 25.4	48.9 58.8 23.1	202.1 278.6 13.3	38.4 47.3 12.7
5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	437.6 437.6 NC	60.5 60.5 NC	365.8 395.8 25.95	52.8 58.1 4.01	305.9 338.9 14.88	42.9 46.8 3.25	227.0 275.7 12.65	34.0 39.9 2.77
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	444.9 444.9 NC	68.2 68.2 NC	379.8 425.0 22.75	57.0 62.1 4.01	336.6 378.8 17.90	50.1 54.9 3.81	225.6 275.9 14.54	32.5 40.5 3.05

NC: Not calculated.

The activity of enzyme catalase decreased with the progress of stress-induced ageing duration (Table 4). The chemical treatment of leaves with ascorbic acid alleviated the ageing-induced rapid loss of catalase.

Table 4. Effect of ascorbic acid (A.A.; 100 µg/ml) on the changes in catalase activity (ΔOD×Tv / t × v) in leaf discs of six wild plant taxa during dark-induced ageing.
Treatments and recording of data are the same as in Table 1.

Plant taxa	**Treatments (µg/ml)**	**Hours after leaf ageing**			
		0	**48**	**96**	**144**
1. *Enkianthus deflexus* var. *acuminatus*	Control A.A. LSD(P=0.05)	57.4 57.4 NC	49.4 51.0 1.4	39.5 46.8 2.0	31.21 40.1 2.1
2. *Gaultheria hookeri*	Control A.A. LSD(P=0.05)	51.6 51.6 NC	43.4 49.3 1.9	36.9 41.0 2.2	29.8 34.2 1.5
3. *G. stapfiana*	Control A.A. LSD(P=0.05)	50.5 50.5 NC	38.9 56.5 3.01	28.7 38.2 2.05	21.5 29.5 0.19
4. *G. trichophylla* var. *ovata*	Control A.A. LSD(P=0.05)	54.4 54.4 NC	42.1 50.0 2.10	32.0 46.2 2.01	26.7 31.8 2.21

5. *Vaccinium nummularia*	Control A.A. LSD(P=0.05)	48.8 48.8 NC	35.5 41.8 3.12	28.7 36.6 2.01	22.9 28.8 2.11
6. *V. glauco-album*	Control A.A. LSD(P=0.05)	70.8 70.8 NC	58.9 65.4 4.92	42.5 56.7 3.88	29.8 45.9 2.85

NC: Not calculated.

Senescence of detached leaves started immediately after the separation from other plants and occurs at a rapid rate with the progressive increase of catabolic activities and these ultimately result in death and decay of leaves. Literature on the senescence pattern and the effect of hormones on regulation of senescence in terrestrial plants are rather scanty (Biswas and Ghosh, 1999; Pati and Bhattacharjee, 2003; Pati, 2007).

Results of this investigation clearly reveal that during the dark induced ageing period of zero to 144 hours of the detached leaves of *Enkianthus deflexus* var. *acuminatus*, *Gaultheria stapfiana*, *G. hookeri*, *G. trichophylla* var. *ovata*, *Vaccinium glauco-album* and *V. nummularia*, the loss of chlorophyll, proteins, insoluble carbohydrates, RNA and DNA occur at a rapid rate. In IAA treated leaf samples the same trend of declining was recorded but the magnitude of loss was found to be much less than control samples. Numerous reports exist in the literature that during all types of senescence loss of some vital macromolecules like chlorophyll and proteins take place which is due to their degradation and /or subdued rate of biosynthesis (Woolhouse, 1967; Leopold and Kriedemann, 1975; Leopold, 1980; Sabatar 1984). Any chemical or external agents possessing the property to maintain the chlorophyll and protein levels during senescence are regarded as senescence retardants (Beevers, 1976; Biswas and Ghosh, 1999; Maity *et al*, 2000).

In this investigation ascorbic acid-induced partial arrestation of the rapid loss of chlorophyll, protein, insoluble carbohydrates, RNA and DNA is indicative of the senescence deferral action of ascorbic acid. Further corroboration regarding the significant the role of the experimental pretreating agent on senescence retardation in the six wild plant taxa can be made from some other biochemical studies like analysis on the enzyme activity catalase as well as the levels of soluble carbohydrate. Catalase is regarded as a scavenzer enzyme (Fridovich, 1976) and higher activity of this enzyme is the index of plant vigour. Rapid loss of catalase activity is indicative of the declining of plant potential (Bhattacharjee & Choudhury, 1986; Biswas and Ghosh, 1999 and Bhattacharjee, 2001; Pati, 2007).

In this investigation the chemical-induced retention of catalase activity during dark-induced detached leaf senescence is indicative of the retardation of senescence. Again, rapid increase of soluble carbohydrate during senescence is mostly due to the damage of cell membrane and subsequent leaching of sugar from the cells. In this study, the rapid increase of sugar in detached leaves of control samples and arrestation of higher leaching of sugar in ascorbic acid-treated samples is indicative of the efficacy of ascorbic acid on the maintenance of the membrane integrity at least to a certain extent. Possibly this chemical-induced maintenance of membrane integrity caused to check the rapid leaching of sugar from the cells. Hence, the sugar levels were found to be much less in the chemical treated leaf samples.

4. CONCLUSION

Considering all the biochemical parameters it can be concluded that ascorbic acid is a potent growth promoter for maintenance of membrane integrity as well as arrestation of overall senescence of the detached leaves of the plant taxa analysed. However, although the ongoing of senescence process during leaf ageing is inevitable, ascorbic acid treatment can retard faster rate of senescence efficiently in some plant species. Thus, ascorbic acid can be considered as a potent senescence deferral agent at least in case of six wild plant ericoid taxa analysed in this investigation.

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