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EFFECT OF ABIOTIC STRESS ON THE PHYSIOLOGY AND ANDROGRAPHOLIDE CONTENT IN *Andrographis paniculata* Nees.

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Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture
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2004

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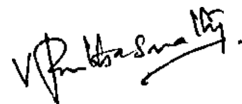
DECLARATION

I hereby declare that this thesis entitled "**Effect of abiotic stress on the physiology and andrographolide content in *Andrographis paniculata* Nees.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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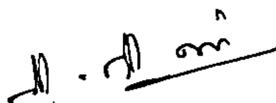

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ACKNOWLEDGEMENT

I wish to extend my profound gratitude and indebtness to **Dr. Roy Stephen,** Assistant professor and chairman of Advisory committee for his constant encouragement rendered to me throughout the course of my study. I owe my flow of thanks to him for his helpful criticism, ever willing help, friendly approach and affection which have greatly facilitated the present investigation.

Grateful and sincere thanks are due to **Dr. M.M. Viji,** Assistant professor and Head of the Department of Plant Physiology for her expert advice, critical comments and constructive perusal of the manuscript.

I am particularly grateful to **Dr.B.T. Krishnaprasad** and **Dr. R. V. Manju,** Department of Plant Physiology, for their valuable guidance and whole hearted guidance throughout the investigation.

My grateful acknowledgements to **Dr. M.G. Purushothama**, Scientist, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for his expert advice, kind help and whole hearted cooperation shown on towards me during my Northern blotting analysis. I also thank my friend, Mr. Rajesh for doing statistical analysis.

I express my profound gratitude to Dr. Babu Mathew, Dr. Anil kumar of department of Agronomy and Dr. Jessy Kutty, Assistant professor, Department of Plantation crops, for whole hearted technical help during andrographolide quantification.

I wish to express my sincere and profound gratitude and obligations to Mr.Haridass for his timely help and valuable advices during the course of this investigation

I also express my sincere thanks to my classmates Jaganathan, Tamilvel, Palanikumar, Sekar, Suresh, Manuel, Jidesh, and

seniors Suthan, Navaz, Srinivasan, Subramanian for their constant help during the course of my research work.

I thank **ICAR** for granting me the Junior research fellowship **(JRF).**

From deep with my heart I express my sincere and profound gratitude to my father, mother, sister and brother and relatives for their constant encouragement and moral support.

Above all I bow before the Almighty for all the blessings showered upon me.

V. Parthasarathy

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LIST OF ABBREVIATIONS

%	Per cent
BSA	Bovine Serum Albumin
^0C	Degree Celsius
Cat	Catalase
CER	Carbon dioxide Exchange rate
CBB	Coomassie Brilliant Blue
Chl	Chlorophyll
CoA	Coenzyme A
cm	Centimetre
cDNA	Complementary DNA
DAS	Days after Sowing
DAP	Days after Planting
DOXP	1-Deoxy-D-Xylulose 5-Phosphate
Day^{-1}	Per day
EC	Electrical Conductivity
et al.	And others
Fe	Iron
FC	Field Capacity
Fig,	Figure
FW	Fresh weight
g	Gram
HMGR	3-Hydroxy -3-Methylglutaryl CoA Reductase
LAD	Leaf Area Duration
kg	Kilogram
m	Metre
μ	Micro
MEP	2-C-Methyl-D-Erthritol 4-Phosphate
mmhos/cm	millimolhs per centimetre
mg	Milligram
Mn	Manganese
NAR	Net Assimilation Rate
nm	Nanometre
PCR	Polymerase Chain Reaction
PX	Peroxidase
RGR	Relative Growth Rate
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SOD	Superoxide Dismutase
SLA	Specific Leaf Area

Introduction

1. INTRODUCTION

Plants produce a broad spectrum of secondary metabolites and the dictionary of natural products has about 85,000 entries for secondary metabolites (Verpoorte, 2000). Plant secondary metabolites represent an enormous value from economical point of view since most of them are used as speciality chemicals such as drugs flavors, fragrances, insecticides and dyes. Of all drugs used in western medicine, about 25% is derived from plants. It is estimated that 80% of the world population depends on traditional medicinal plants for their primary health care (Verpoorte, 2000).

Past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products worldwide. International market for medicinal plants is over US$ 60 billion per year, which is growing at the rate of seven per cent. The plant medicinal usage by the American public alone have shown an increase from just 3 per cent of the population in 1991 to over 37 per cent in 1998 (Brevoort, 1998). India is exporting herbal material to the tune of Rs. 446.3 crores and it is estimated to become Rs. 3000 crores by 2005 (Ghosh, 2000).

Kerala, with its abundance of luxuriant flora is the only state in the country where Ayurveda continues to be practiced in its purest form. There are about 853 pharmaceutical units in Kerala out of which a dozen of them consume about 50 per cent of the total plant based raw material produced and used for drug manufacture. The demand for ayurvedic medicine in the state is growing at a rate of 10-12 per cent per annum. The projected export marketing of herbal medicine is worth US$ 5 billion per year by 2010 in Kerala (Kumar, 2003).

Andrographis paniculata Nees. an erect annual herb of 30-100 cm height is one of the 19 species of Andrographis belonging to the family Acanthaceae. It has been in use in Indian system of medicine since time immemorial and is one of the most widely used plants in ayurvedic formulations (Saxena *et al.,* 1998). It is called 'Kalmegh' in Bengali, 'Kirayat' in Hindi, 'Kiriyattu' or 'Nelavepu' in Malayalam. The medicinally active compounds of this plant are

'Andrographolide'-very bitter compounds known as diterpene lactones found in the aerial parts of the plant. The plant has been reported to posses antipyretic, analgesic, antihepatotoxic, antihistamic, antibacterial, anti-inflammatory properties and also used in the treatment of fever, cold, cough, tonsillitis, bronchitis, osteogynea, artheralgia, hypertension, snake bite etc (Singh *et al.*, 2001). Estimated demand of the drug in India is 1000 tons/ year and the rate of this plant in the Indian market is Rs. 18000/ton with ayurvedic and homeopathic drug manufacturers as major users (Saxena *et al.*, 1998).

The medicinal actions of plants are unique to particular species or groups owing to taxonomically specific secondary metabolites. The secondary compounds have a pivotal role in the ecophysiology of plants and the ecological functions have some bearing on their potential medicinal effects for humans. Plant secondary metabolites have protective actions in relation to abiotic stress also (Briskin, 2000). Hence variations in the environmental factors should alter the secondary metabolism. Although active phytochemicals have been identified, in general, many pathways for the biosynthesis of specific medicinal compounds and the biotic and abiotic factors regulating their production remain unclear. Variation in phytochemical content due to environmental effects upon plant secondary metabolism in the plant could cause a significant concern on quality control in herbal manufacturing practices.

Hence understanding how the environmental factors affect phytomedicinal chemical production will be of great importance towards optimizing field growth conditions for medicinal plants for maximal recovery of phytomedicinal chemical. It is also unknown, the extent to which, the levels of phytochemical production are determined by genetic potential versus environmental modulation. Such information will help us to understand how plants respond to environmental stresses affecting their survival and how humans can alter plants metabolism to favour the synthesis of a particular metabolite of medicinal or economical value. In this scenario, the present investigation was proposed to study the environmental, biochemical and molecular mechanisms which up regulate or

Review of Literature

down regulate the gene expressions and enzyme activities that control the synthesis of secondary metabolites, with the following objectives.

1. To study the effect of stress factors on the growth and andrographolide content.
2. To analyze the physiological and biochemical changes due to stress in *Andrographis paniculata.*
3. To assess the expression of key gene involved in secondary metabolite production in *Andrographis paniculata.*

Review of Literature

2. REVIEW OF LITERATURE

Plant secondary metabolites have a pivotal role in the ecophysiology of plants with mainly protective actions in relation to stresses (Kaufman *et al.*, 1999). The phytomedicinal contents of different species are affected by environmental factors and developmental stages of the plants (Li and Mazza, 1999). It is also important to know that the extent to which, the levels of secondary metabolite production by medicinal plants are determined by genetic potential versus environmental modulation. In this context the present study is aimed to analyse the effect of different abiotic stresses given at different developmental stages on growth and andrographolide content of *Andrographis paniculata* Nees. literature pertaining to the effect of abiotic stress on physiological, biochemical and molecular aspects in related species are reviewed in this chapter.

2.1 BIOMETRIC CHARACTERS

2.1.1 Plant Height

Destined to reside in the habitats of germination, plants are frequently exposed to unfavorable environmental conditions. Extreme temperature, drought, and salinity, greatly affect plant growth, development, and productivity. Plant height is the one of the primary variables affected by stress (Taiz and Zeiger, 2003).

Positive influence of shade on plant height was reported in colocasia (Pramela, 1990). In ginger an increasing trend in plant height with increasing shade intensity was reported (Ancy, 1992; Beena, 1992; Babu, 1992 and Sreekala, 1999).

Mint species when grown as an intercrop in sugarcane under sub-tropical climate, increased plant height was observed compared to the sole crop (Singh *et al.*, 2002).

O. kilimandscharicum and O. minimum) with saline solution, indicated that the high salinity levels significantly reduced herb yield (Salem *et al.*, 2001). A reduction in dry matter accumulation under salinity was reported in crops such as

Jana and Ghildyal (1996) observed that the plant height of rice progressively increased when soil water content varied from unsaturation to flooding. In cucumber plants combination of UV-B radiation and water stress resulted in decreased plant height, when compared with the control (Yang *et al.*, 2000). Plant height in rice was decreased remarkably with the decrease of irrigation regime (Pradhan *et al.*, 2003).

2.1.2 Dry Matter Production

Pramela (1990) reported highest dry matter production at 25 per cent shade level in colocasia compare to open condition. When *Panax quinquefolium* plants were grown under a black polypropylene shade canopy, there was rapid stem growth and dry matter production (Bailey and Stathers, 1991).

In the effects of shade on growth of jarrah (*Eucalyptus marginata*), it was observed that plant dry weight declined in response to shade (Stoneman and Dell, 1993). In arrowroot, total dry matter production was higher in intercrop compared to open space grown crop (Maheswarappa, 1997).

Andrographis paniculata plants were grown under varying light intensities (15, 30, 45 or 100% of full sunlight) for a year. The total DM production of the plants grown under full light was always less than that of the plants grown under 15 per cent light (Pratima, 1998).

A reduction in dry matter production was noticed under low light condition in rice (Janardhan and Murthy, 1980). In *Aloe vera*, it was observed that plants grown under full sun produced twice the total dry mass than those grown under partial shade (Paez *et al.*, 2000).

The effects of irrigation on some Ocimum species (*O. basilicum, O. kilimandscharicum and O. minimum*) with saline solution, indicated that the high salinity levels significantly reduced herb yield (Salem *et al.*, 2001). A reduction in dry matter accumulation under salinity was reported in crops such as vetiver (Belligno *et al.* 2002) and pepper (Pascale, 2003). Salt grown

catharanthus exhibited low biomass production compared to the control (Karadge and Gaikwad, 2003)

In Malus, drought caused an increase in root biomass production (Atkinson *et al.,* 2000). In sugarcane, Ramesh (2000) found a reduction in dry matter content under moisture stress condition.

Soyabean plants grown under 70 per cent shade did not show any reduction in dry matter (Erilson and Whitney, 1984).

2.1.3 Leaf Area

Sudden environmental changes bring about rapid and often transient changes in leaf elongation rate. Such environmental changes include variation in light intensity, humidity, salinity and soil water potential. The speed of the response suggests that it is the changes in leaf water status that drive the initial changes in elongation rate. The role of cell turgor or tissue water status in determining leaf growth rates was well emphasized.

In ginger, increased leaf area under reduced light intensity was reported (Ravisankar and Muthuswamy, 1988; Ancy, 1992).

In forage grasses, response to reduced light (shade) include larger leaves with fewer mesophyll cells and stomata per unit leaf area, more intercellular air space, higher leaf area ratio (LAR), and reduced specific leaf weight (SLW) (Kephart, *et al.,* 1992). In a study to find the effect of light intensity and quality on the growth and quality of Korean ginseng (*Panax ginseng* C.A. Mayer), it was observed that as plant density increased the leaf area per plant decreased.

In ginger minimum leaf area was noticed in plants grown under open condition (Sreekala, 1999). In wheat under saline condition, leaf area was reduced adversely (Dinesh *et al.,* 2000). Leaf area was significantly reduced in tree species grown in open and 40 per cent shade conditions compared to open condition (Netshiluvhi, 1999).

In sorghum cultivars, leaf area decreased in all the lines studied under water stress (Rao, 1999). In cucumber plants combination of UV-B radiation and water stress resulted in decreased leaf area compared with the control (Yang *et al.*, 2000).

Morales *et al.* (2002) found that leaf area was reduced under salinity in tomato.

2.1.4 Leaf Area Duration (LAD)

Leaf area duration (LAD) expresses the magnitude and persistence of leaf area or leafiness during the period of crop growth. It reflects the extent or seasonal integral of light interception and has been shown to correlate highly with yield.

Ipor and Price (1992) studied the response of *Paspalum conjugatum* to shade and found that LAD increased by shading. LAD was highest in 25 per cent shade followed by 50 and 75 per cent shade and was the lowest under open condition in ginger (Babu, 1992; Ajithkumar, 1999).

2.1.5 Specific Leaf Area (SLA)

Specific leaf area is the leaf area per unit leaf dry weight and the inverse of specific leaf weight is a reduction of the thickness. Species achieve the highest net CO_2 exchange rate per unit leaf area (CER) from the minimum leaf material are efficient (Rawson, 1987). On the contrary, Dornhoff and Shibles (1976) showed a negative relation in soyabean.

SLA has its positive and negative role through its link with photosynthetic volume (Charles, 1982). Thus lower SLA means more layer of mesophyll cells (Dornhoff and Shibles, 1970) and greater leaf area (Pettigrew *et al.*, 1993). The genotypic differences in photosynthetic rate per unit leaf area have been correlated with genotype differences in SLA in many species including alfalfa (Pearce *et al.*, 1969): soyabean (Dornhoff and Shibles, 1976 and in peanut (Rao and Wright, 1994).

2.1.6 Relative Growth Rate (RGR)

Relative growth rate (RGR) expresses the dry weight increases in a time interval in relation to initial biomass.

Both absolute biomass gain and relative growth rate (RGR) have significant positive correlations with water potential, stomatal conductance and leaf carbon content. Multiple regression analysis identified water potential and stomatal conductance as the factors, which contributed most to the observed variation of absolute biomass gain and RGR (Costa and Rozana, 2000).

At different growth phases significantly higher RGR was recorded at open, 20 and 40 per cent shade (Ajithkumar, 1999). Paez *et al.* (2000) reported that RGR increased at all growth intervals under shade in tomato.

Contrary to these reports a negative trend in RGR were also observed due to shade and such negative trend were reported in *Erythrina variegata* Lam (Muthchelian *et al.*, 1989), pepper (Jung *et al.*, 1994) and rice (Viji, 1995).

Effects of artificial shading on growth of *Trema micrantha* (Ulmaceae) seedlings revealed a decrease in relative growth rate caused by reductions in net assimilation rate rather than leaf area ratio (Valio, 2001).

In cucumber plants combination of UV-B radiation and water stress resulted in decreased relative growth rate, and increased specific leaf mass compared with the control (Yang *et al.*, 2000). In canola, the relative growth rate was decreased under the water stress treatment (Dehshiri *et al.*, 2001).

In wheat, relative growth rate was affected adversely under salinity (Dinesh *et al.*, 2000).

2.1.8 Net Assimilation Rate (NAR)

Net assimilation rate is the assimilation capacity per unit leaf area. NAR is not constant with time but shows an ontogenic downward drift with plant age

which might be accelerated by an unfavorable environment and due to natural shading.

An increase in NAR with increase in shade intensity was reported in coco yams (Valenzuela, 1990), and in ginger (Ancy, 1992; George, 1992). Babu (1992) found that during the first phase of study (60-120 DAP), the high NAR was recorded from open but at second phase (120-180 DAP), maximum NAR was obtained from 25 per cent shade level.

Janardhanan and Murthy (1980) observed that in rice under low light condition, NAR was reduced. Similar results were reported in cogon grass Patterson (1980), *Crotalaria speeta* (Patterson, 1982), cucumber (Smith *et al.*, 1984), *Paspalum cojugatum* (Ipor and Price, 1992), Tannia (Pushpakumari and Sasidhar, 1992) and Pepper (Jung *et al.*, 1994).

Several reports indicated that NAR was not influenced by increase in shade intensity in crops like cocoa seedlings (Gopinathan, 1981), ginger and turmeric (Lalithabai, 1981), *Mentha arvensis* (Duriyaprapan and Brittun, 1982), Greater yams (Pushpakumari and Sasidhar, 1992) and ginger (Ajithkumar, 1999).

In sorghum cultivars, NAR decreased in all the lines studied under water stress (Rao, 1999). In cucumber plants, combination of UV-B radiation and water stress resulted in decreased net assimilation rate compared with the control (Yang *et al.*, 2000). In canola cultivars net assimilation rate was decreased under water stress treatment (Dehshiri *et al.*, 2001) and similar results were reported in okra (Ashraf *et al.*, 2002).

In wheat with salinities of 0, 5, 10, 15 or 20 dS/m net assimilation rate (NAR) was reduced with increasing salinity level (Dinesh *et al.*, 2000).

2.2. PHYSIOLOGICAL PARAMETERS

2.2.1 Stomatal Conductance

The application of moderate water stress to plants inhibited photosynthesis by 75 per cent, which was attributable at least in part due to CO_2 limitation by

stomatal closure because there were large decreases in stomatal conductance and transpiration.

In *Cryptantha flava*, shading did not reduce stomatal conductance proportionately to photosynthesis, which led to decreased water use efficiency for plants under shrubs (Forseth *et al.*, 2001).

In *Lycium barbarum* when the concentration of NaCl was less than 0.6%, the leaf intercellular carbon dioxide concentration (Ci) was decreased, while the limiting value of stomata (Ls) increased (Zheng *et al.*, 2002).

2.2.2 Stomatal Frequency

In *Impatiens flanaganae*, an increase in stomatal abnormalities was noticed in leaves of plants grown under high light intensities. These abnormalities include degradation of guard cells, super imposed and contiguous stomata, stomata with persistent intervening walls, cytoplasmic connections, single guard cells, guard cells without pores and persistent stomatal cells (Lall and Bhat, 1996).

A study carried out to investigate the degree of leaf wetness and its capacity to retain water droplets in relation to leaf morphological characteristics of *Valleriana jatamansi* grown under open and shade habitats revealed that leaves developed in open habitats had less wettability but higher capacity to retain water droplets and more number of stomata than those in shade habitats (Pandey and Nagar, 2002).

2.2.3 Membrane Stability Index

In wheat, the membrane stability index was brought down by salinity (Sairam *et al.*, 2000) and water stress (Sairam *et al.*, 2001).

2.2.4 Chlorophyll Content

Higher plants contain chlorophyll a the major, yellow–green pigment, chlorophyll b (blue-green) accessory pigments and several additional forms of

chlorophylls. Several works demonstrated rapid change in chlorophyll content to internal as well as external factors.

In shade grown plants of *Andrographis paniculata*, plants had more chlorophyll per unit dry weight (Pratima, 1999).

In sorghum, water stress enhanced the chlorophyll content (Satbhai et *al.*, 1998). A decrease in the chlorophyll–a, chlorophyll–b and total chlorophyll content was observed with the increase in the sodium chloride concentration compared to control plants in *Spirodeca polyrhiza* (Khan and Panda, 2000).

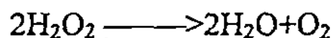
Chlorophyll a/b ratios were similar across both sun and shade levels for *Vinca major* L (Demmig and Adams, 1992).

A significant reduction in chlorophyll 'b' was observed due to water stress in *Grevellia robusta* by (Nautiyal *et al.*, 1993) and similar result was reported in tea (Rajasekar *et al.*, 1991).

2.3 BIOCHEMICAL PARAMETERS

2.3.1 Peroxidase Activity (EC.1.11.1.7)

Peroxidase isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma (PX) and thylakoid membrane (PX) in chloroplasts, the microbody (mPX), and the cytosol (cPX).



$$2H_2O_2 \xrightarrow{PX} 2H_2O + O_2$$

The plants under stress enhance the activities of enzymatic antioxidant especially peroxidase (Scandalios, 1993). The transcript level of pea peroxidase increased four fold in response to drought stress and was dramatically enhanced (fifteen fold) following recovery from stress (Mittler and Zilinskas, 1992). The increased peroxidase activity was reported in water deficit stressed Marigold (Karup *et al.*, 1994) and same result was reported in sugarcane (Yang *et al.* 1995).

The transgenic tobacco with higher expression of peroxidases had increased tolerance to photo oxidative damage (Mano *et al.*, 1994).

In tobacco plants, salinity lead to two to three fold increase in the activity of SOD and peroxidases (Vancamp *et al.*, 1996). An increase of peroxidase activity was noticed in salt grown *Lycopersicon pennelli* (Shalata and Tal, 1998), Arabidopsis (Tsugene *et al.*, 1999), spinach (Yoshimara *et al.*, 2000) and rice (Mandal and Singh, 2000). Zhang and Kirkham (1996) reported a decrease in activity of peroxidase in sunflower and sorghum seedlings under water stress.

In Spinach leaves, the activities of the peroxidase isoforms decreased as the intensity of light increased, despite the fact that the transcription and protein levels of these isoforms were not altered (Yoshimura *et al.*, 2000).

2.3.2 Catalase Activity (EC.1.11.1.6)

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Catalase is one of the first enzymes to be isolated in a highly purified state. Multiple forms of catalase have been described in many plants. Plants have three isoforms of catalase termed cat-1, cat-2 and cat-3 that are on separate chromosomes and are differentially expressed and independently regulated. The cat-1 and cat-2 are localised in peroxisomes and the cytosol, whereas cat-3 is mitochondrial.

Catalase is very sensitive to light and has a rapid turnover rate similar to that of the D1 protein of PSII, as a result of light absorption by the heme. Stress conditions, which reduce the rate of protein turnover, such as salinity, heat shock or cold cause the depletion of catalase activity. This may have significance in the plant's ability to tolerate the oxidative components of these environmental stresses. Catalase is critical for maintaining the redox balance during oxidative stress. Such control may not be limited to peroxisomal H_2O_2 production.

Moran *et al.* (1994) have reported that catalase activity was decreased in pea plants under water stress.

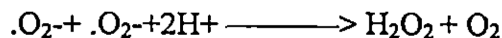
Catalase activity was not affected by mild drought in sorghum (Zhang and Kirkham, 1996).

The inhibition of new synthesis of catalase in the presence of sodium chloride has been documented by radioactive labeling (Hertwing *et al.*, 1992). In cotton, a higher constitutive concentration of catalase was found in salt tolerant lines than salt sensitive lines (Gossett *et al.*, 1994a). In Tobacco, NaCl and $CuSO_4$ triggered differential alteration in the transcript levels of the different Cat genes. The NaCl induction of Cat transcripts was correlated with increased catalase activity (Willekens *et al.*, 1997). NaCl was found to enhance catalase, activities in *Gossypium hirsutum* callus tissue (Gossett *et al.* 1994b). Allen (1995) has shown that catalase deficient mutant of tobacco had higher sensitivity to light and salt stresses. Benesides *et al.* (2000) reported that the catalase activity was increased under salinity in *Solanum tuberosum.*

2.3.3 Superoxide Dismutase (EC.1.5.1.1.)

Superoxide dismutases are a family of metalloenzymes that catalyze the disproportionation of O^{-2} radicals (super oxide) into H_2O_2 and O_2, and play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments. SODs are distributed in different cell loci, mainly chloroplasts, cytosol, and mitochondria.

Superoxide Dismutase



$$.O_2-+ .O_2-+2H+ \longrightarrow H_2O_2 + O_2$$

Mn-SOD: mitochondria

Fe-SOD: Chloroplast

CuZn-SOD: Chloroplast and cytosol

Therefore, the activity of this enzyme determines the relative proportions of the two constituents of the Haber-Weiss reaction that generates hydroxyl

radicals. Since SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress. There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn - SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found in the cytosol, others in the chloroplasts of higher plants. The Fe-SOD isozymes are often not detected in plants, but when detected, Fe-SOD is usually associated with the chloroplast compartment.

Several forms of SOD have been cloned from a variety of plants. It has been shown that SOD activity was increased in cells in response to diverse environmental and xerobiotic stresses like high light, salinity, water logging and drought. Apparently, each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments. This role may be served by unique lipid peroxidation products from each organelle that diffuse from the site of oxidative damage to the nucleus where they would enhance transcription of specific SOD genes (Buchanan *et al.*, 2000).

Gogorcena *et al.* (1995) reported that SOD activity decreased in drought stressed pea nodules and a similar reduction was reported in rice (Boo and Jung, 1999).

In water stressed wheat plants, SOD activity remained unchanged (Bartoli *et al.*, 1999). Water stress did no influence on SOD activity in sorghum (Zhang and Kirkham, 1996)

In *Quercus robue* L, salt stress caused an increase in SOD activity (Schmer *et al.*, 1995) and same resulted was reported in *Pisum sativum* (Olmos *et al.*, 1994), *Lysopersicon pennelli* (Shalata and Tal 1998), *Arabidopsis* (Tsugane *et al.*, 1999) and rice (Kaminaka *et al.*, 1999; Tanaka *et al.*, 1999). It has been demonstrated that salt tolerant cotton, barely, tomato, and wild beet, exhibited higher constitutive and induced levels of SOD as compared to their salt

translocation, and respiration. Among the soluble carbohydrates, sucrose and fructans have a potential role in adaptation to these stresses. Hanson and Hitz, (1982) have reported that sugar content decreased under water stress.

In shade *grown Murcott tangor,* the starch concentration was increased in leaves (Raveh *et al.*, 2003).

In chickpea, water stress reduced total soluble sugars and starch (Yadav *et al.,* 1996). In sorghum, water stress resulted in reduced sugars (Satbhai *et al.*, 1998). During water stress, the soluble sugar content in *Populus euramericana* was decreased (Marrison *et al.*, 2002).

The effects of water stress at preflowering stage was studied in moth bean and reported that water stress significantly reduced starch and increased sugar content (Garg *et al.*, 2001). Soluble sugars and starch were increased by water stress in wheat plants (Hamada, 2003).

Uday *et al.* (2002) reported an increase in concentration of starch under high salinity in isabgol.

There was a decrease in sucrose and no increase in reducing sugar content under salinity in *Cenchrus penniseliformis* (Ashraf, 1997). In Rice (*Oryza sativa*) germinated under salt-stressed conditions, Mandal and Singh, (2000a) found that the starch and sugar content decreased under saline conditions.

2.3.6 Free Amino Acids

Total free amino acids indicate the physiological health status of the tissue (Sadasivam and Manickam, 1996). Drought stress induced an ArgE-related polypeptide and caused a massive accumulation of the free amino acid citrulline in the leaves (Amici *et al.*, 1989). Cry *et al.* (1989) reported that total soluble free amino acid level rose as water stress increased in three species of *Picea mariana*, white spruce and jack pine. Drought stress caused organ specific increase in free amino acids in *Populus tremuloides* (Griffin *et al.*, 1991). In sorghum, water stress enhanced the content of free amino acids (Satbhai *et al.*, 1998). During drought,

levels of citrulline and arginine increased up to 49 and 11% of total free amino acids in the watermelon leaves (Kawasaki *et al.*, 2000).

Total free aminoacids were reported to be higher in salt tolerant than in salt sensitive lines of safflower (Ashraf and Tufail, 1995) *Eruca sativa* (Ashraf, 1994) and *Lens culinaris* (Hurkaman, 1991). Yadav *et al.* (1997) showed that free amino acids were variously accumulated under stress in chickpea. Free amino acids have been reported to accumulate in higher amounts under salinity stress in lentil (Mansour, 2001)

Plants subjected to salt stress had an increase in the concentration of total amino acids in legumes (Garg *et al.*, 1996). In chickpea, water stress also reduced free amino acids content (Yadav *et al.*, 1996).

When rice (*Oryza sativa*) seeds were germinated under salt-stressed conditions, Mandal and Singh, (2000b) found that the free amino acid content was decreased under saline conditions. Uday *et al.* (2002) reported a decrease in free amino acids under salinity in Isabgol.

2.3.7 Total Soluble Protein Content

Total leaf soluble protein exhibited a 2.5 fold increase in *Vinca major* and nearly a 20 fold increase in pumkin when shifted from shade to full sunlight (Logan *et al.*, 1998).

The effects of water stress at preflowering stage was studied in moth bean (Garg *et al.*, 2001) and found that increasing water stress significantly reduced soluble protein content. Soluble protein content increased significantly under water stress in palmosa (Fatima *et al.*, 2002).

A higher content of total soluble protein had been observed in salt tolerant than in salt sensitive cultivars of safflower (Ashraf and Fatima, 1995), finger millet (Uma *et al.*, 1995), and rice (Parek *et al.*, 1997). Ashraf and Waheed (1993) reported that leaf soluble proteins decreased due to salt stress in lentil. Ashraf and Leavy (1999) reported that total soluble protein increased due to salt stress in all cultivars of wheat.

Ashraf and Fatima (1995) found that salt tolerant and salt sensitive accessions of safflower did not differ significantly in leaf soluble protein content. Similarly comparison of salt tolerant and wild population with cultivated *Melilotus indica* and *Eruca sativa* showed that the salt tolerant populations did not differ from salt sensitive populations in terms of soluble protein content at varying salt levels in the growth medium (Ashraf, 1994).

2.3.8 Secondary Metabolite

A common consequence of most abiotic and biotic stress is that they result, at some stage of stress exposure, they result an increased production of reactive oxygen species. Apart from the xanthophyll cycle, photorespiration and other changes in metabolic activity which may protect the chloroplasts from oxidative damage. A number of enzymatic and nonenzymatic antioxidants are present in chloroplasts that control oxygen toxicity. Carotenoids, α-tocopherol (α-T, vitamin E), ascorbate (Asc, vitamin C), and glutathione help to maintain the integrity of the photosynthetic membranes under oxidative stress. Besides, some plants contain secondary metabolites which display high antioxidant properties in vitro. Secondary metabolites have potent antioxidant properties that have received considerable attention in food science and biomedicine (Kaufman, 1999).

In shade grown *Uncaria rhynchophyll*, Kawazoe *et al.* (1989) have reported that plant growth rate and alkaloid contents in the hooks increased with the degree of shade. In *Atropa korupensis*, shade significantly increased the amounts of michellamine B (Thomas *et al.*, 1994). Boldo (*Peumus boldus Mol*) leaves grown in the shade had higher essential oil and alkaloid contents than those in full sunlight (Vogel *et al.*, 1996).

Shade-grown *Aloe arborescence* and *A. saponaria* contained less anthocyanins and carotenoids than those grown in the field (Lee *et al.*, 1996).

Fully and partially exposed tea in open light had approximately two fold high levels of chlorogenic acid than shaded leaves (Grace *et al.*, 1998). Mole *et al.* (1998) explained that high light activates the phenyl propanoid pathway.

In *Centella asiatica*, 50 per cent shading of plants resulted in higher yields of herbage and asiaticoside (Shalini *et al.*, 2000). At understorey of four tree species, *Acumen klaineana, Marquesia excelsa, Paraberlinia bifoliolata,* and *Xylopia hypolampra.* A survey done at tropical rain forest in gabon revealed that the quantity of biologically active molecules of plants were more in shade than open grown plants (Downum *et al.*, 2001).

In shade grown *Plantago lanceolata*, the contents of aucubin and acteoside were extremely lower in plants grown in the shade (Tamura, 2001).

Rosemary plants have species –specific antioxidant, abietane diterpenes that increased during drought (Munne-Bosh *et al.*, 1999).

The effect of irrigation on some Ocimum species (*O. basilicum, O. kilimandscharicum O. lanceolata and O. minimum*) with saline solution of 750, 1500, 2400 and 3600 ppm along with the control of 315 ppm on its growth yield, and oil composition were analysed and found that some of the essential oil constituents increased with increasing salinity levels as methyl eugenol and camphor in the *O. basilicum* and *O. kilimandscharicum*, and linalool in all studied species. Eugenol and α-terpineol decreased with increasing salinity levels (Salem *et al*, 2001). The essential oil content increased under salt stress (Yao *et al.*, 2002).

2.3.9 Total Phenol Content

Among the environmental factors that influence phenolic metabolism, light intensity has a particularly a strong effect .For instance, flavonoids exhibit a fluence rate dependence, typical of high irridance response of plant photo morphogenesis (Begg and Wellman, 1994). High irradiation induces flavonoids (particulary Kaempferol derivatives) and sincepate esters in Arabidopsis and Isoflavonoids and Psovalens in other species (Lois, 1994).

Shetty (1991) has reported accumulation of polyphenols in the leaves of *Achrosticum aeurum* grown in low salt regimes. It had been reported that the

highly evolved halophytes like Mangroves are rich in polyphenols (Joshi, 1976), which helps their survival under stress conditions.

Antioxidant properties of phenols arise from their reactivity with hydrogen as electron donors and from the ability to chelate transition metal ions (Rice – evans *et al.*, (1997). According to Takahama and Oniki, (1997) the condensed tannins (flavonols) as measured by high performance liquid chromatography were 1000 times higher in normal *Pseudocorus rhizomes* than in those of stressed one. The effect of stress on flavonals content (a decrease after 35 days of treatment) suggests their participation in the antioxidant defense in normal ones.

2.3.10 3-Hydroxy -3-Methylglutaryl CoA Reductase (EC.1.1.1.34)

Metabolic specialization is a characteristic trait during cell differentiation. Metabolic activities associated with specific cells are essential for growth and survival of plant cells. In some cell types, metabolic specialization may lead to the synthesis of secondary metabolites. Plant isoprenoids are one of the diverse group of plant secondary metabolites and their biosynthesis is a complex, multi branched pathway. The reactions leading to specific isoprenoid end products emerge from a central pathway in which acetyl coenzyme A (CoA) is converted via mevalonate and isopentyl disphosphate(Fig.1.).

Higher plants contain another pathway called DOXP/MEP pathway for isoprenoid synthesis in the plastids. This pathway is alternative with respect to the classical Mevalonate pathway, starts with condensation of pyruvate and glyceraldehyde -3-phosphate which yields 1-deoxy-D-xylulose 5-phosphate (DOXP) which will be converted to isopentenyl diphospahte (IPP) and eventually to isoprenoids or thiamine and pyridoxal. Subsequent reactions of this pathway involve transformation of DOXP to 2-C-methyl-D-erythritol 4-phosphate (MEP), which on condensation with CTP forms 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME). Then CDP-ME is phosphorylated to 4-Disphocytidyl-2-C-methyl-D-erythritol 2 –phosphate (CDP-ME2P) and to 2-C-Methyl-D-erythritol -2-4-cyclodiphosphate (ME-2, 4cPP), which is the last known intermediate of the DOXP/MEP pathway (Fig. 2).

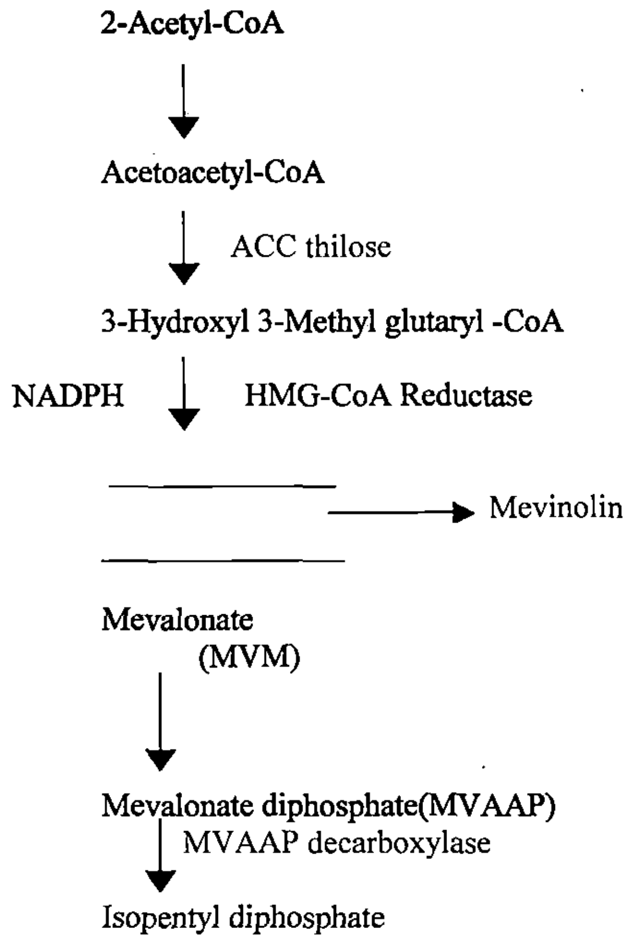


Fig.1. Acetate- Mevalonate (AC-MVA) path way (In cytoplasm) (McGavery and Croteau, 1995)

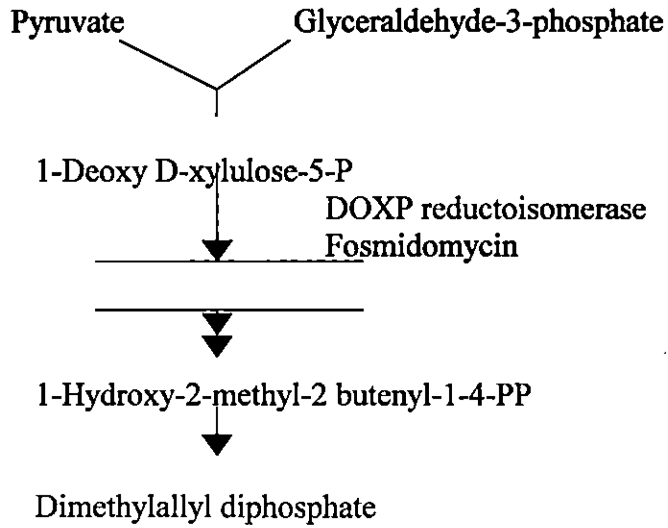


Fig. 2. Non-Mevalonate pathway (In Plastids)

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This novel pathway appears to be involved in the biosynthesis of carotenoids, phytols, isoprene, mono, di, tetraterpenes and plastoquinone whereas the mevalonate pathway is responsible for formation of sterols, sequiterpenes and triterpenes. Several isoprenoids were found to be of mixed origin suggesting that some exchange and cross talk between these two pathways of different biosynthetic origin takes place (Fig. 3).

In classical pathway, the synthesis of mevalonate, catalysed by the enzyme 3-hydroxy-3 methylglutaryl CoA (HMGR) is considered the first rate-limiting step in plant isoprenoid biosynthesis. HMGR from many plant species has been characterized, and its activity has been found to be regulated by different physiological and environmental stimuli, such as phytohormones, light, wounding, pathogen attack, feed back mechanisms and endogenous protein factors. The occurrence of multiple genes encoding HMGR in higher plants has been characterized and two genes are reported in Arabidospsis, three genes have been reported in *Hevea brasilienis* (Chye *et al.*, 1992) and four genes are present in tomato (Cramer *et al.*, 1993). Larger HMGR gene families have been reported in wheat (Aoyagi *et al.*, 1993) and potato (Stermer *et al.*, 1994).

The pattern of expression of individual genes encoding HMGR has been established in a limited number of plants. In *Hevea brasiliensis* three differentially expressed genes encoding HMGR have been identified (*hmg1, hmg2 and hmg3*). The Hevea *hmg* 3 gene is constitutively expressed, where as *hmg1* is expressed predominately in the laticifers and is inducible by ethylene (Chye *et al.*, 1992). In tomato, HMGR is encoded by four genes that are differently expressed during development and in response to stress (Cramer *et al.*, 1993).

Arabidopsis contains two differentially expressed *hmg* genes, *hmg1* and *hmg2*. The *hmg1* mRNA has been detected in all parts, whereas the presence of *hmg2* mRNA has been shown to be restricted to young seedlings, roots and inflorescence (Chye *et al.*, 1992). Although the two encoded proteins (*hmg1* and *hmg2*) have the same structural organization and intercellular location, hmg2

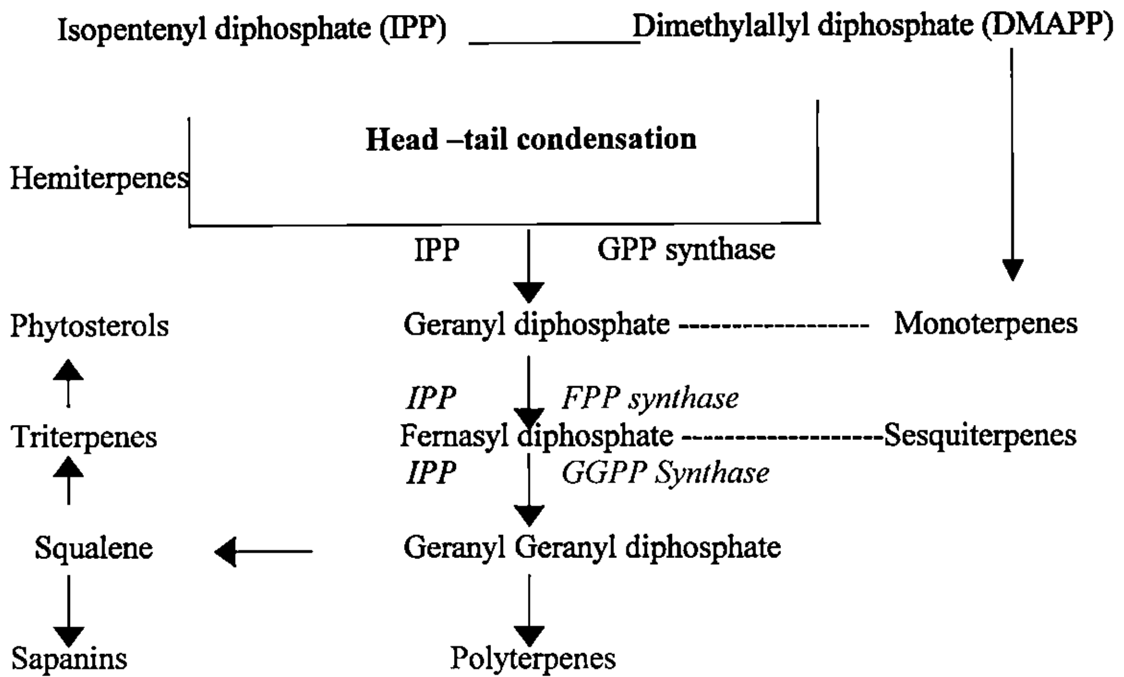


Fig. 3. Cytoplasmic AC –MVA pathway for IPP synthesis and its restricted role in the biosynthesis of terpenoids (McGavery and Croteau, 1995).

represents a divergent form of the enzyme that has no counterpart among the plant hmgs identified.

When dark-grown pea seedlings received 5 minutes of irradiation with red light and then returned to darkness, the HMGR activity contained in the plastid fraction doubled after 1.75 hours (Wang *et al.*, 1982). In maize seedlings, germinated in the dark both root and shoot HMGR activity were 1-5 folds higher relative to activities of seedlings in white light (Moore and Oishi, 1993). Plant HMGR was negatively regulated by red light in pea seedlings (Brooker and Russell, 1979). In *Arabidopsis thaliana,* HMGR is negatively regulated by light (Learned, 1996).

Materials and Methods

3. MATERIALS AND METHODS

A pot culture experiment was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani to study the effect of abiotic stresses on growth and andrographolide content, physiological and biochemical changes and to asses the patten of gene expression in *Andrographis paniculata*. The work was implemented by conducting three separate experiments. For the first experiment, *Andrographis paniculata* plants were raised in plastic bags and different abiotic stresses namely, water stress, salinity were imposed for a period of 30 days to plants aged 60 days, 90 days and 120 days of planting. The plants were grown at three different light levels during entire crop season. Andrographolide was quantified after this stress period. Five treatments from each shade level were selected for detail to study the influence of abiotic stresses on growth aspects, physiological and biochemical changes, andrographolide content and to assess the expression of key genes involved in secondary metabolite production in *Andrographis paniculata.*

3.1 EXPERIMENTAL DETAILS

3.1.1 Location

The pot culture experiment was conducted at College of Agriculture, Vellayani, and situated at $8^{0}5$'N latitude and $76^{0}9$'E longitude at an altitude of 29 m above mean sea level. The soil of Instructional Farm, which was used for the study, was red loam and comes under rhodic haplastox. The soil was acidic with a pH of 5.2 and with an EC of 0.002 dsm^{-1}.

3.1.2 Season

The experiments were conducted from December 2003 to May 2004 and weather data during the period is given in appendix I.

Plate 1. ***Andrographis paniculata*** **pot culture under open condition**

Plate 2. ***Andrographis paniculata*** **pot culture under 50 per cent shade condition**

Plate 3. ***Andrographis paniculata*** **pot culture under 75 per cent shade condition**

3.1.3 Planting Material

Andrographis paniculata seeds were collected from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

3.1.4 Layout of the Experiment

The Experiment was laid in CRD. The numbers of replications were four.

3.1.5 Outline of Technical Programme

The whole research program was classified into three experiments. Each experiment was carried out in different shade levels *viz.*, open, 50 per cent and 75 per cent shade. The treatments included three levels each of salinity, growth stage, and water stress. The water stress levels were 100 per cent, 60 per cent and 30 per cent of field capacity. The salinity levels were control (EC = 0.002), 3 and 6 mmhos/cm. The growth stages were 60, 90 and 120 days of sowing respectively. These treatment combinations are given in the table 1. Here, O denotes open condition, F denotes fifty per cent shade and H denotes seventy five Per cent of shade. G denotes growth stages where G1 denotes 60 days, G2 denotes 90 days and G3 denotes 120 days of sowing. W denotes water stress of 100, 60, and 30% field capacity denoted by W1, W2, and W3 respectively. Similarly salinity levels S1, S2 and S3 denote control, 3 mmhos /cm, and 6 mmhos/cm.

3.1.6 Preparation and Planting

The experiment was conducted in pots of 10 litres volume filled with potting mixture consisting of farm yard manure, sand and soil in ratio of 1:1:1. The seeds were sown in tray. After 20 days they were transplanted into pots.

3.1.7 Artificial shading

Three shade levels (open, 50 % and 75%) were provided by using high-density polyethylene nets purchased from Kerala Agro Industries Corporation which was spread over pandals.

Table 1. Representation of treatment combinations

Sl. No	Open	50% Shade	75% Shade
1	OG1S1W1	FG1S1W1	HG1S1W1
2	OG1S1W2	FG1S1W2	HG1S1W2
3	OG1S1W3	FG1S1W3	HG1S1W3
4	OG1S2W1	FG1S2W1	HG1S2W1
5	OG1S2W2	FG1S2W2	HG1S2W2
6	OG1S2W3	FG1S2W3	HG1S2W3
7	OG1S3W1	FG1S3W1	HG1S3W1
8	OG1S3W2	FG1S3W2	HG1S3W2
9	OG1S3W3	FG1S3W3	HG1S3W3
10	OG2S1W1	FG2S1W1	HG2S1W1
11	OG2S1W2	FG2S1W2	HG2S1W2
12	OG2S1W3	FG2S1W3	HG2S1W3
13	OG2S2W1	FG2S2W1	HG2S2W1
14	OG2S2W2	FG2S2W2	HG2S2W2
15	OG2S2W3	FG2S2W3	HG2S2W3
16	OG2S3W1	FG2S3W1	HG2S3W1
17	OG2S3W2	FG2S3W2	HG2S3W2
18	OG2S3W3	FG2S3W3	HG2S3W3
19	OG3S1W1	FG3S1W1	HG3S1W1
20	OG3S1W2	FG3S1W2	HG3S1W2
21	OG3S1W3	FG3S1W3	HG3S1W3
22	OG3S2W1	FG3S2W1	HG3S2W1
23	OG3S2W2	FG3S2W2	HG3S2W2
24	OG3S2W3	FG3S2W3	HG3S2W3
25	OG3S3W1	FG3S3W1	HG3S3W1
26	OG3S3W2	FG3S3W2	HG3S3W2
27	OG3S3W3	FG3S3W3	HG3S3W3

3.1.8 Treatment Details

For imposing salinity, the soil from Instructional Farm, Vellayani was taken as control. The salinity was maintained by adding sodium chloride solution. The plants were irrigated equally to keep at the field capacity and were kept at respective shade levels. Sequential monthly sowing was undertaken during December, January and February months so that the treatments could be imposed at 60, 90, and 120 days of planting and simultaneously during the same season to reduce variability due to the season. Prior to the treatment, all the pots were irrigated at evening and left over night to bring them to field capacity. The exposed part of the soil on the pot was completely covered with the broken pieces of pot. The drainage holes were closed with cement. The weight of the individual pot with soil, pot pieces and plant was recorded with the help of an electronic digital top loading weighing balance. The water holding capacity of the soil was calculated gravimetrically. All the pots in each shade level were grouped into three sets with one set at FC and others at 60% and 30 % FC. The weight of pots at 60% and 30% was calculated based on the water holding capacity of the soil equation given below using the equation given below.

Pot weight at 100% FC (W100) $=A+B+Q_{100}$

Pot weight at 60% FC (W100) $=A+B+Q_{60}$

Pot Weight at 30% FC (W100) $=A+B+Q_{30}$

Where, A=soil dry weight with pot

B=Broken pot pieces (spread on each pot surface to prevent evaporation)

Where as Q_{100}, Q_{60} and Q_{30} =Quantity of water present at 100% FC, 60%FC and 30% FC respectively.

The plants were kept at respective soil moisture level by adding water daily, which was lost by transpiration in the previous day. The transpiration loss was calculated by weighing the pots daily. Plants were kept at respective soil moisture level by adding same quantity of water, which was lost by transpiration

during previous day calculated by weighing the pots daily with it difference from the respective reference weight.

3.2 BIOMETRIC OBSERVATIONS

Sampling was done before and after the 30 stress day's treatment for biometric observation. Primary growth parameters recorded were

a) Plant height

b) Leaf area

c) Dry weight

From the above primary values the following parameters were computed.

a) Leaf area Duration (LAD)

b) Relative Growth Rate (RGR)

c) Net Assimilation Rate (NAR)

d) Specific Leaf Area (SLA)

3.2.1 Sampling procedure for SLA

1. From each pot, fully expanded third leaf (from main stem apex) was collected. Leaflets were separated and the petioles were discarded and area was measured.

2. Leaflets were dried at 80°C for 2 days and the dry weight was taken.
   SLA was calculated using the formula.

$$SLA\ (cm^{-2}.g^{-1}) = \text{Leaf area/ dry weight}$$

3.2.2 Dry Matter Production (DMP)

Whole plants were uprooted and dried to constant heat at 80 ± 5^0C in hot air oven. The sum of the dry weight of all the plant parts gave the total dry matter yield.

3.2.3 Net Assimilation Rate (NAR)

NAR was calculated using the procedure given by Williams (1946). NAR is expressed as g m^{-2} day^{-1}.

$$NAR = \frac{(W_2-W_1)\ (LogeW2-LogeW1)}{(T_2-T_1)(L_2-L_1)}$$

Where, W_2 – Total dry weight of plant g m^{-2} at time T_2

W_1 – Total dry weight of plant g m^{-2} at time T_1

(T_2-T_1) = Time interval in days

L_2 – Leaf area at time T_2

L_1 – Leaf area at time T_1

3.2.4 Relative Growth Rate (RGR)

RGR was calculated as per the method described by Williams (1946) and expressed as mg^{-1} day^{-1}.

$$RGR = \frac{\log_e w_2 - \log_e w_1}{t_2-t_1}$$

Where, w_1 and w_2 are total dry weights per plant at time t_1 and t_2 respectively and (t_2-t_1) is the time interval in days.

3.2.5 Leaf Area Duration (LAD)

LAD was calculated using the formula suggested by Hunt (1983)

$$LAD = \frac{(L_1 + L_2) \times (t_2-t_1)}{2}$$

Where

L_1 – LA at first stage

L_2 – LA at second stage

(t_2-t_1) – Time interval between two month stages

3.3 PHYSIOLOGICAL PARAMETERS

3.3.1 Estimation of Chlorophyll

100 mg of leaf sample was taken. It was chopped into pieces and added 5 ml of DMSO (Dimethyl sulfoxide) and left over night. Then read at 645 and 663 nm. The chlorophyll a, chlorophyll b, and total chlorophyll were calculated using the formulae given below and expressed in mg of pigments per gram of fresh leaf as per Sadasivam and Manikam (1976)

$$\text{Total chlorophyll} = 20.2\,(\text{OD at } 645) + 8.01\,(\text{OD at } 663) \times \frac{V}{W \times 1000}\ \text{mg/g}$$

$$\text{Chlorophyll a} = 12.7\,(\text{OD at } 663) - 2.69\,(\text{OD at } 645) \times \frac{V}{W \times 1000}\ \text{mg/g}$$

$$\text{Chlorophyll b} = 22.9\,(\text{OD at } 645) - 4.68\,(\text{OD at } 663) \times \frac{V}{W \times 1000}\ \text{mg/g}$$

3.3.2 Stomatal Frequency

Stomatal count refers to the number of stomata per unit area of leaf. A thick mixture of thermocol and xylene was prepared and this was smeared on both surface of leaves and allowed to dry. It was pealed gently after drying and the peel was observed under microscope and counted using a 40 x objective and 10 x eyepiece. The field of the microscope was measured using a stage micrometer and stomatal frequency per unit area was calculated.

Stomatal frequency = Number of stomata/Area of the microscopic field.

3.3.3 Stomatal Conductance

Stomatal conductance was measured using the ΔT Porometer of company Delta T Devices and expressed as cm/s

3.4. BIOCHEMICAL PARAMETERS

3.4.1 Ascorbic Acid

Ascorbic acid was determined by volumetric method as proposed by Sadasivam and Manikam (1996).

One gram of the leaf material was homogenized with 4 % oxalic acid and centrifuged at 5000rpm for 10 min. Th volume of the supernatant was made upto 25 ml. To 5 ml of the aliquot, added 10 ml of 4 % oxalic acid and titrated against 2,6-dichlorophenol indophenol dye. 5 ml of the working standard containing 100μg/ml of ascorbic acid was also titrated for reference value. The amount of ascorbic acid was expressed as $\mu g\ g^{-1}$ FW.

3.4.2 Estimation of Andrographolide Content

Andrographolide content was estimated as per the procedure of Gained *et al.*,(1963). One gram of finely powdered leaf material was taken. 100ml of benzene was added and kept in the water bath for 1 hour at 50-60 degree Celsius. It was kept for 3-4 hours at room condition and centrifuged at 8500 rpm with hot benzene repeated for removing chlorophyll completely. Then it was dried and 10 gram of kiselguhr was added. The mixed powder was extracted in a soxhlet apparatus with 250 ml pure chloroform for 3-4 hrs. After that, it was redissolved in 40 ml of methyl alcohol. Then it was kept for some time to settle the kiselguhr powder. The methyl alcohol solution of *Andrographis paniculata* extract was then read at 223nm absorbance in spectrophotometer and the per cent

of andrographolide content was calculated from standard curve. The pure andrographolide from Sigma Company was used as the standard.

3.4.3 Membrane Stability Index (MSI)

Fully expanded leaves of different treatment combinations were excised with their petioles intact in water and allowed to regain turgidity in distilled water for 45 minutes. The leaves were then allowed to live wilt under shade condition.

After leaves have lost 40 per cent to 60 per cent weight, leaf punches of 1 cm diameter were taken. These punches were washed for 1 to 2 minutes to leach out their solutes from the cut ends and blotted on clean paper. Leaf punches were incubated in water at room temperature for 3 hours. Then leakage of solutes in this bathing medium was estimated by recording its absorbance at 273 nm. This was referred as the initial leakage of solutes.

Then samples were incubated in hot water bath (100°C) for 15 minutes. After suitable dilution the absorbance of the bathing medium was again read at 273 nm to indicate the finial absorbance due to the leakage of total solutes contained in the tissue. The per cent leakage of solutes which was a direct reflection of the extent of loss of membrane integrity was calculated as per following

$$\text{MSI} = \frac{\text{Initial absorbance of bathing medium}}{\text{Final absorbance of bathing medium}} \times 100$$

3.4.4 Total Soluble Protein

Total soluble protein of leaf was estimated using simple protein – dye binding method of Bradford (1976) using Bovine Serum Albumin as the standard. This method is based on the principle that the Coomassie Brilliant Blue (CBB) G-250 binds to protein and the protein dye complex has a higher extinction coefficient thus leading to a great sensitivity in

measurement of protein. This binding of dye to protein is a very rapid process (approx .2 min). The method is devoid of interferences by other soluble compounds.

100 mg of CBB G-250 was dissolved in 50ml of 95 per cent ethanol. To this solution 100 ml of 85 per cent (W/V) ortho-phosphoric acid was added. The resulting solution was diluted to the final volume of 200 ml with distilled water.

One gram of leaf material was ground to a thin paste and soluble protein was extracted with 10 ml of phosphate buffer (pH 7.8) containing 1mM EDTA, 2 per cent (W/W) PVP. The extract was centrifuged in cold (4^0 C) at 10,000 rpm for 10 minutes. To 50-µ l of the supernatant 4 ml of Bradford reagent was added and mixed well. The absorbance of the solution was recorded at 595 nm, after two minutes and within 30 minutes using spectrophotometer.

The protein content was calculated using the BSA standard in the range of (10-100 microgram). The protein content was expressed both as mg/g fresh weight

3.4.5 Estimation of Starch

Starch was estimated by the method of Sadasivam and Manickam (1996). 0.5 g of leaves was homogenized in 80% ethanol. The absorbance at 630 nm was read. The starch content was calculated using glucose standard and multiplied with a factor of 0.9 to arrive at the starch content. The starch content was expressed as mg per gram F $w^{-1}$of leaf

3.4.6 Estimation of Reducing Sugar

Reducing sugar was estimated by the method of Sadasivam and Manickam (1996). 0,5 g of leaves was homogenized in 80% ethanol. The absorbance was read at 510nm. Sugar content was found using the glucose as standard.

3.4.7 Estimation of Free Amino Acid

Free amino acid content was estimated by the method of Sadasivam and Manickam (1996). Readings were taken at 570 nm absorbance against blank. For running standard, Leucine was used.

3.4.8 Estimation of Phenol

Total phenol was estimated by the method of Sadasivam and Manickam (1996). Readings were taken at 650 nm absorbance against blank. For running standard, catechol was used as standard.

3.5 ANTIOXIDANT ENZYMES

3.5.1 Estimation of Catalase (EC 1.11.1.6)

Catalase activity was determined by Luck (1974) method. One gram of the leaf sample was extracted in 0.067M phosphate buffer (pH 7.0). A Known volume of the extract was added to the cuvette containing 3ml H_2O_2 and 0.16 M PO_4 buffer. The time taken for change in absorbance (Δt) at 240 nm was recorded for calculating the enzyme units per ml of extract. All operations were carried out at about at 0 to 4 ^{0}C

3.5.2 Estimation of Peroxidase (EC.1.11.1.7)

Peroxidase activity was determined by adopting the method of Malik and Singh (1980).

One gram of leaf material was extracted in 0.1 M phosphate buffer (pH 7.0). A known volume of the extract was added to an experimental cuvette containing 3 ml of 12.3 mM phosphate buffer and 0.03 ml guaiacol reagent and then 0.03ml of H_2O_2 solution was added rapidly and increase in absorbance at 436 nm was recorded. This Δt was used to calculate the enzyme activity. The enzyme activity was expressed as enzyme units per ml of extract. All operations were carried out at 1 to 4°C.

3.5.3. Estimation of Superoxide Dismutase (EC.1.15.1.1)

Superoxide dismutase, activity was assayed by monitoring the inhibition of the photochemical reduction of Nitroblue terazolium (NBT) according to the method of Beau and Fridovich (1971). For total, 1ml reaction mixture of the super oxide dismutase assay contained 1 M Phosphate buffer (pH 7.6),0.1 mM EDTA,50 mM sodium carbonate,13mM Methionine,75 μ Molar NBT, 2 μ molar Riboflavin and one ml aliquot of enzyme extract. The reaction mixture was illuminated for 15 minutes at light intensity mixture of 350 μ mole per meter square per second. One unit of superoxide dismutase activity was the amount of NBT reduced, which was monitored at 560 nm.

Absorbance of reference blank = X

Absorbance of sample = Y

[(X-Y)/ X] x100 = Z% inhibition

50 Per cent inhibition = Z/50= A unit

i.e., 50μl enzyme extract yield A unit.

1000 μl yields A/50 x1000=B unit

B/ Protein Value= SOD assay value (Enzyme units mg^{-1} protein ml^{-1})

3.6. Statistical Analysis

The experiments were analyzed in complete randomized block design (CRD). AGRES stat package was used for analysis.

3.6.1 Grouping of Treatment Combinations

Grouping of treatment combinations was done based on frequency treatments around mean value. Grouping was classified into three divisions.

First division= <Mean- Standard error

Second division = Mean ± Standard error

Third division = >Mean+ Standard error

Then entry of treatment corresponding values was made and frequency distribution of treatment was expressed in per cent.

3.7 GENE EXPRESSION

Gene expression studies were carried out at Ranjiv Gandhi Centre for Biotechnology Thiruvananthapuram.

3.7.1 RNA Isolation

3.7.1.1 DEPC Treatment of Glasswares and Milli-Q water

DEPC was added to Milli-Q water to a final concentration of 0.02%, mixed well using a magnetic stirrer and kept overnight. The residual DEPC was destroyed by autoclaving the treated water at 121°C at 1.05 kg/cm^2 for 20 min. Glasswares and plastic wares used for RNA isolation and cDNA synthesis were treated with DEPC water and autoclaved as mentioned above. Glasswares for reagent preparations were baked further at 180°C in a hot air oven.

3.7.1.2 Total RNA Isolation from A. paniculata leaves

Total RNA was isolated from 1 g leaf tissue using Trizol™ reagent. One gram of young leaves was frozen in liquid nitrogen. Frozen tissue was ground to fine powder in an RNase free mortar using pestle. Ten mL Trizol™ reagent was added to the ground tissue and was kept at room temperature for 5 min. The homogenate was transferred to a 50 mL RNase free centrifuge tube. The sample was centrifuged at 12000g for 10 min at 4°C. The supernatant was transferred to an RNase free centrifuge tube and 200 µL of chloroform was added per 1 mL Trizol™ reagent used. The sample was centrifuged at 12000g for 15 min at 4°C. The aqueous phase was collected in a new centrifuge tube and 5 mL isopropanol was added and kept at room temperature for 10 min to precipitate the total RNA. The sample was centrifuged at 12000g for 10 min at 4°C. The supernatant was discarded and the pellet was washed in 10 mL of 75% ethanol by centrifugation at 7500g for 5 min at 4°C. After centrifugation ethanol was decanted and the RNA

pellet was dried at room temperature. The RNA pellet was dissolved in 500 µL of nuclease free water and stored at -70°C.

3.7.1.3 DNase I treatment of total RNA

The total RNA was treated with DNase I to remove any traces of genomic DNA present. The reaction mix contained total RNA, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 5 units of DNase I set to a final volume of 200 µL. The reaction was incubated at 37°C for 10 min followed by incubation at 65°C for 15 min to denature DNase I. The treated RNA was extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1) by centrifugation at 10,000 g for 10 min at room temperature. To the aqueous phase, 20 µL of 3 M sodium acetate and 200 µL of isopropanol were added and incubated at room temperature for 10 min. The sample was centrifuged at 10,000g for 10 min at room temperature. The RNA pellet was washed with 1 mL of 75% ethanol at 7500g for 5 min. The pellet was air dried and dissolved in 100 µL of nuclease free water. The quality and integrity of total RNA was checked in a 2% agarose gel by loading 5 µL samples along with loading dye.

3.7.1.4 Quantitation of Total RNA

Total RNA was quantitated spectrophotometrically at 260 nm. The concentration of RNA in 1 µL of the RNA sample was calculated by the following equation.

$$\frac{40 \text{ x O.D } A_{260} \text{ x dilution factor}}{1000}$$

First strand cDNA was synthesized from mRNA by reverse transcription using M-MuLV Reverse Transcriptase (M-MuLV RT). The reaction mix was prepared for 50 µL volume with 4 µg of mRNA, 1 X M-MuLV RT buffer, 200 units of M-MuLV RT (Promega), 5 mM dNTP mix, 5 units of RNasin (RNase inhibitor) and 2.0 µg oligo dT_{18} primer. Total RNA, Oligo dT_{18} primer and nuclease free water were added to a nuclease free microfuge tube and denatured to remove secondary

structure from RNA by incubating at 65°C for 10 min and snap cooling on ice. The sample was spun down to remove any condensed reagent at the microfuge lid and rest of the components were added to the denatured RNA. RT reaction was incubated at 42°C for 90 min in a water bath followed by heat inactivation of the enzyme at 75°C for 15 min on a heating block. Sample was stored at -20°C.

3.7.1.5 Relative Quantitative RT-PCR

Relative quantitative RT-PCR was carried out to determine the expression level of the *Aphmgr* genes in the selected treatments of the plant. Total RNA was isolated from different tissue samples of *A. paniculata* using Trizol™ Reagent and DNase I treated. Total RNA was isolated from plants under open at 60 DAS with 6mmhos/cm salinity and 60% field capacity from plants grown at 50% shade at 90 DAS with 6mmhos/cm salinity and 30% FC and also from a control plant at 50% shade without any salinity or water. The isolated RNA was checked in 2% agarose gel and quantitated by spectrophotometry. The first strand cDNA reaction consisted of a final volume of 100 μL containing 20 μL 5 X RT buffer, 3 μL (300 μM) of 10 mM dNTP mix, 3 μL (~700 ng) of oligo dT primer, 20 units of RNase inhibitor and 400 units of MMuLV-RT. All reagents except total RNA were prepared as a master mix and split into respective reactions with different RNA samples. Ten μg of total RNA made up to 20 μL with nuclease free water was incubated at 70°C for 10 min and quick chilled on ice. Eighty μL of master mix containing all other reagents was added to each of the the denatured total RNA. The reagents were mixed well by gentle tapping, and the cDNA synthesis was carried out at 42^{0}C for 1 h followed by heat denaturation of the enzyme by incubation of the sample at 70^{0}C for 10 min.

The 30 μL RT-PCR reaction contained 2 μL of cDNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of forward and reverse primers, 200 μM dNTP mix and 1 unit of *Taq* DNA polymerase. A master mix of the components except the cDNA was made and dispensed separately to the PCR tubes followed by the addition of the cDNA and over layered with 25 μL of mineral oil. PCR reactions were carried out the annealing temperature and details of the primers are given in

table (Table 2). The PCR cycles were determined based on the log phase of the amplification for each gene. *β*-actin was used as the internal control for amplification. Ten μL of the reactions were checked on 1.2% agarose gel. The PCR was repeated using two individual cDNA preparations.

Table 2. Primers, Tm and Number of PCR cycles used in relative quantitative RT-PCR of the *Aphmgr, Apdxs* and *β*-actin.

Gene	*Aphmgr1*	*Aphmgr2*	*β-actin*
Forward primer	SKW10	SKW10	ACF
Reverse primer	HMGRR1	HMGRREV1	ACR
Tm ^{0}C	63	60	50
No. of PCR Cycles	26	35	32

HMGRR1 – 5' TTG GAG CGG AAC AAC TAT TTC AGC T 3'

HMGRREV1 – 5' AGG AGG GAA AAA GAA AAA AGG CCC A 3'

ACF – 5' TCC ATA ATG AAG TGT GAT GT 3'

ACR – 5' GGA CCT GAC TCG TCA TAC TC 3'

Results

4. RESULTS

A Pot culture experiments was conducted to study the effect of abiotic stresses, identify treatments, which have a positive influence on andrographolide content under different shade (50% and 75 %) and open condition. Activities of the scavenging enzymes such as catalase, peroxidase, superoxide dismutase and other physio and biochemical parameters were collected to relate these parameters to andrographolide content. These pot culture experiments were done by imposing 27 treatments under fifty, seventy per cent shade levels and under open condition. Several biochemical parameters like starch, sugar, total phenol and total free amino acids were also recorded in selected treatments, which were selected based on andrographolide content. The inter relationship between various parameters under different combinations were also computed.

4.1. BIOMETRIC CHARACTERS.

4.1.1 Plant Height (cm)

Significant variation in plant height was observed among the treatment combinations in open and different shade conditions. Data on the effect of stress on the plant height of *Andrographis paniculata* is presented in Table 3. The plant height increased with shade level and growth stage. Water stress and salinity stress reduced the plant height in all shade levels.

In open condition, the mean height recorded was 21.60 cm and the range in height was 5.1to 30.37 cm (Table 3). The plant height was reduced significantly in the treatment combinations OG1S1W3, OG1S2W3, OG1S3W2, and OG1S2W3. The reduction of plant height compared to mean value was 67 to 76 per cent. The treatment combinations of OG2S1W1, OG2S1W2, OG2S2W1, OG2S2W2, OG2S2W3, OG2S3W1, OG2S3W2, OG3S1W1, OG3S1W2, OG3S1W3, OG3S2W1, OG3S2W2, OG3S2W3, OG3S3W1, OG3S3W2 and OG3S3W3 did not show any influence on plant height. The frequency distribution of treatment combinations under open condition indicates that 33 per cent of

Table 3. Effect of abiotic stress on plant height of *Andrographis paniculata* under different shade condition

Sl. No.	Treatment	Plant height (cm)		
		Open	50% shade	75% shade
1	G1S1W1	12.4	14.6	15.6
2	G1S1 W2	9.6	12.4	13.8
3	G1S1W3	7.8	10.8	10.4
4	G1S2W1	11.8	14.2	15.2
5	G1S2W2	9.6	11.2	12.8
6	G1S2W3	7.6	9.6	10.2
7	G1S3W1	11.2	13.8	13.6
8	G1S3W2	8.6	10.8	11.4
9	G1S3W3	5.1	6.3	7.2
10	G2S1W1	29.1	34.2	36.3
11	G2S1W2	27.3	32.1	34.65
12	G2S1W3	25.8	30.45	33
13	G2S2W1	28.8	33.6	35.85
14	G2S2W2	26.85	31.53	33.6
15	G2S2W3	24.45	30.15	32.7
16	G2S3W1	28.8	32.1	36.15
17	G2S3W2	27.15	28.95	34.2
18	G2S3W3	25.35	28.35	32.1
19	G3S1W1	29	33.625	35.5
20	G3S1W2	27.62	31.5	34
21	G3S1W3	26.75	30.25	32.875
22	G3S2W1	30.37	33	35.25
23	G3S2W2	28.87	32.25	33.625
24	G3S2W3	28.5	30.875	32.75
25	G3S3W1	29.75	32.375	34.75
26	G3S3W2	28.12	30.375	33
27	G3S3W3	26.75	29.875	32.25
	Mean	**21.60**	**24.79**	**26.77**
	CD (5%)	**3.822**	**4.358**	**4.70**

Table 3a: Grouping of 27 treatments and their frequency distribution for plant height of *Andrographis paniculata* under different shade condition

Plant height under open				Plant height under fifty per cent shade			Plant height under seventy five per cent Shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean-SE	<19.69	1,2,3,4,5,6,7,8,9	-9-	<26.62	1,2,3,4,5,6,7 ,8,9	-9-	<24.43	1,2,3,4,5,6,7 ,8,9	-9-
Mean±SE	19.69-23.50	NIl	-0-	22.62-26.96	NIL	-0-	24.43-29.11	NIL	-0-
Mean+SE	>23.50	10,11,12,13,14, 15,16,17,18,19, 20,21,22,23,24, 25,26,27	-18-	>26.96	10,11,12,13, 14,15,16,17, 18,19,20,21, 22,23,24,25, 26,27	-18-	>29.11	10,11,12,13, 14,15,16,17, 18,19,20,21, 22,23,24,25, 26,27	-18-

treatment combinations had plant height of less than 19.69 cm and 67 per cent had more than 23.50 cm (Table 3a).

In fifty per cent shade level, the height of the plant varied between 6.3 to 33.62 cm and the mean value was 24.79cm. The plant height was reduced significantly in the treatment combinations of OG1S2W3 and OG2S3W3. The reduction of plant height due to stress was 61 to 74 per cent compared to mean value. The treatment combinations of FG2S1W1, FG2S1W2.FG2S2W1, FG2S2W2, FG2S2W3, FG2S3W1, FG2S3W2, FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W2, FG3S2W3, G3S3W1 and FG3S3W2 showed no influence of stress .The frequency distribution of treatment combinations under fifty per cent shade condition indicates that 33 per cent of treatment combination had plant height of less than 26.62 cm and 68 per cent had more than 26.96 cm (Table 3a).

The mean plant height of 26.77 cm was recorded under seventy five per cent shade level and the range in plant height was 7.2 to 36.15 cm. The plant height was reduced significantly in the treatment combinations of HG1S1W3, HG1S3W2, and HG1S3W3. The per cent of reduction was 62 to 73 compared to mean value. The treatment combinations HG2S1W1, HG2S1W2, HG2S2W1, HG2S2W2, HG2S2W3, HG2S3W1, HG2S3W2, HG3S1W1, HG3S1W2, HG3S1W3, HG3S2W1, HG3S2W2, HG3S2W3, HG3S3W1, HG3S3W2 and HG3S3W3 showed no influence of stress .The frequency distribution of treatment combination under seventy five per cent of shade level indicate that 33 per cent of treatment combination had plant height of less than 24.43 cm and 67 per cent had more than 29.11 cm (Table 3a).

4.1.2 Dry Matter Production (g plant^{-1})

Significant variation exists for the dry matter production in both shade and open conditions. The data presented in Table 4 represents the effect of stress treatments on dry matter production in *Andrographis paniculata* under different shade and open condition .The mean dry matter production was 37.063 g under open condition and the range in dry matter production was 7.7 to 72 g per plant.

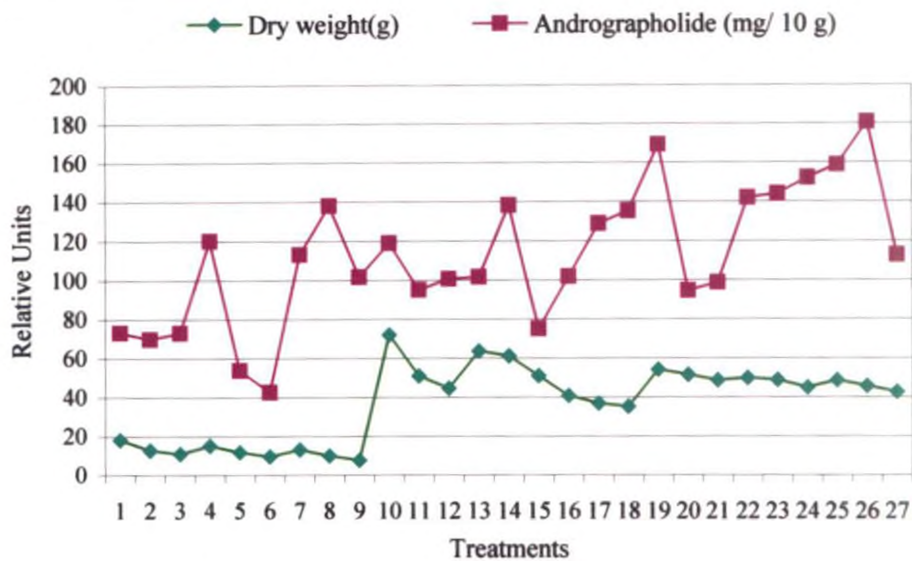


Fig. 4. Relatioship between the andrographolide content and dry weight in open condition

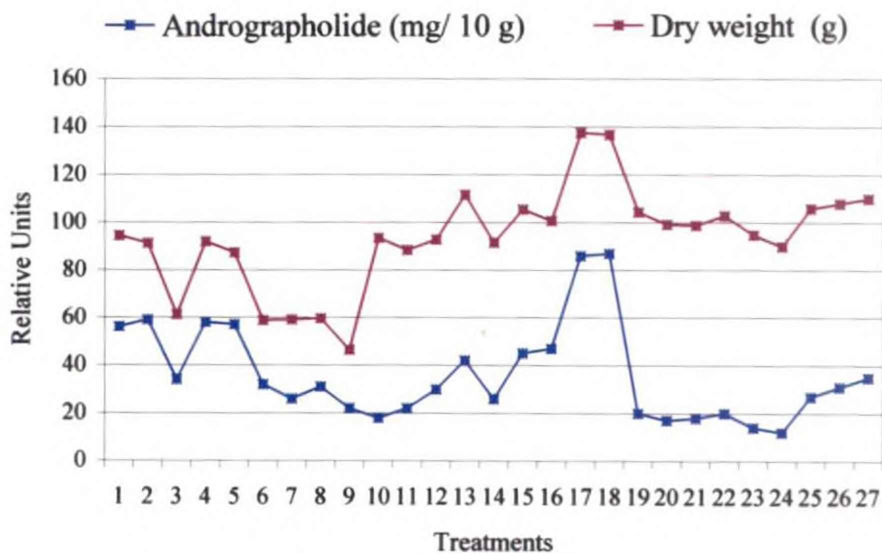


Fig. 5. Relationship between the andrographolide content and dry weight in 50 % shade

Table 4. Effect of abiotic stress on dry weight of *Andrographis paniculata* under different shade condition

Sl. No	Treatment	Dry weight (g plant $^{-1}$)		
		Open	50% shade	75% shade
1	G1S1W1	18.24	38.46	25.55
2	G1S1 W2	12.82	32.28	20.75
3	G1S1W3	10.94	27.29	17.86
4	G1S2W1	15.10	34.02	21.22
5	G1S2W2	11.84	30.41	18.36
6	G1S2W3	9.65	26.76	16.48
7	G1S3W1	13.18	33.01	20.58
8	G1S3W2	9.92	28.58	18.45
9	G1S3W3	7.75	24.39	15.99
10	G2S1W1	71.96	75.52	51.73
11	G2S1W2	50.93	66.48	41.38
12	G2S1W3	44.67	62.74	38.09
13	G2S2W1	63.67	69.61	47.73
14	G2S2W2	61.13	65.58	44.76
15	G2S2W3	50.93	60.41	40.27
16	G2S3W1	40.82	53.67	36.50
17	G2S3W2	36.89	51.60	33.83
18	G2S3W3	35.16	49.74	32.48
19	G3S1W1	54.11	84.40	48.69
20	G3S1W2	51.58	82.18	47.23
21	G3S1W3	48.75	80.79	45.15
22	G3S2W1	49.83	82.86	45.42
23	G3S2W2	48.82	80.84	45.17
24	G3S2W3	45.08	77.99	43.02
25	G3S3W1	48.75	78.90	45.56
26	G3S3W2	45.59	76.95	43.49
27	G3S3W3	42.64	74.95	41.54
	Mean	**37.06**	**57.42**	**35.08**
	CD (5%)	**6.826**	**10.01**	**6.063**

Table 4a. Grouping of 27 treatments and their frequency distribution for dry matter production under different shade condition.

Dry matter production under open				Dry matter production under fifty per cent shade			Dry matter production under seventy per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<33.65	1,2,3,4.5,6, 7, 8,9,	-9-	<52.43	1,2,3,4;5,6,7 , 8,9,17,18	-11-	<32.05	1,2,3,4,5,6,7, 8,9,	-9-
Mean ± SE	33.65- 40.46	Rest of the treatments	-2-	52.43- 62.41	Rest of the treatments	-2-	32.05- 38.10	Rest of treatments	-3-
Mean+SE	>40.46	10,11.12,13,1 4,15,16.19,20, 21,22.23,24,2 5,26,27	-16-	>62.41	10,11,12,13, 14,19,20,21, 22,23,24,25, 26,27,	-14-	>38.10	10,11,12,13, 14,15,19,20, 21,22,23,24, 25,26,27	-15-

The dry matter production was reduced significantly by the treatment combination of OG1S1W2, OG1S1W3, OG1S2W2, OG1S3W3, OG1S3W1, OG1S3W2 and OG1S3W3. The per cent of reduction in dry matter production was 79 compared to the mean value. The frequency distribution of treatment combinations indicates that 33 per cent of treatment combinations had dry matter of less than 33.65 g and 59 per cent had more than 40.46 g (Table 4 a)

In fifty per cent shade condition, the dry matter production of plants varied between 24.38 to 84.40 g per plant with a mean of 57.42. The dry matter production was reduced significantly by the treatment combinations of FG1S2W3, FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG1S3W1, FG1S3W2, and FG1S3W3 and the reduction in dry matter production was 53 per cent compared to mean value The treatment combination of FG2S1W2, FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W2, FG3S3W1, and FG3S3W3 were statistically non significant. The frequency distributions of treatment combinations indicates that 40 per cent of treatment combinations had dry matter of less than 52.43 g and 51.8 per cent had more than 62.41 g (Table 4a).

In seventy five per cent shade condition, the mean dry matter production was 35.08 and the range in dry matter production was 15.99 to 51.73 g per plant. The dry matter production was reduced significantly by the treatment combinations of HG1S1W1, HG1S1W2, HG1S3W2, HG1S2W1, HG1S2W1, HG1S2W1, HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2 and HG1S2W3. The reduction in dry matter production was 40 to 54 per cent compared to mean value. The treatment combination of HG3S1W1, HG3S1W2, HG3S1W3 and HG3S3W1 were statistically non significant. The frequency distribution of treatment combinations indicates that 33 per cent of treatment combinations had less than 32.05 and 56 per cent had more than 38.18 g of dry matter production (Table 4a). The relationship of andrographolide content with total dry matter of plants under open, 50% shade and 75% shade are given in Fig 4, 5, 6 respectively. There was only a week correlation between andrographolide content and plant biomass. The relationship of andrographolide content with total dry matter of plants under open,

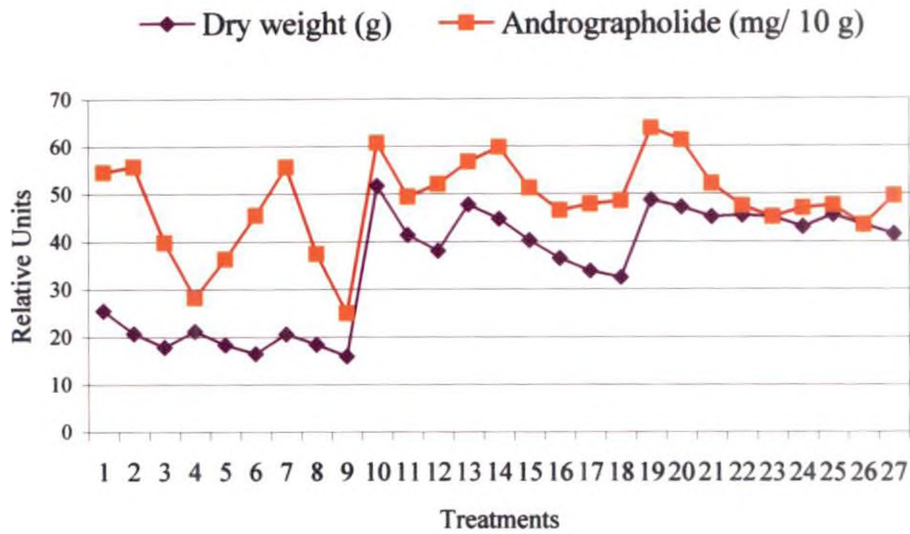


Fig. 6. Relationship between the andrographolide content and dry weight in 75% shade

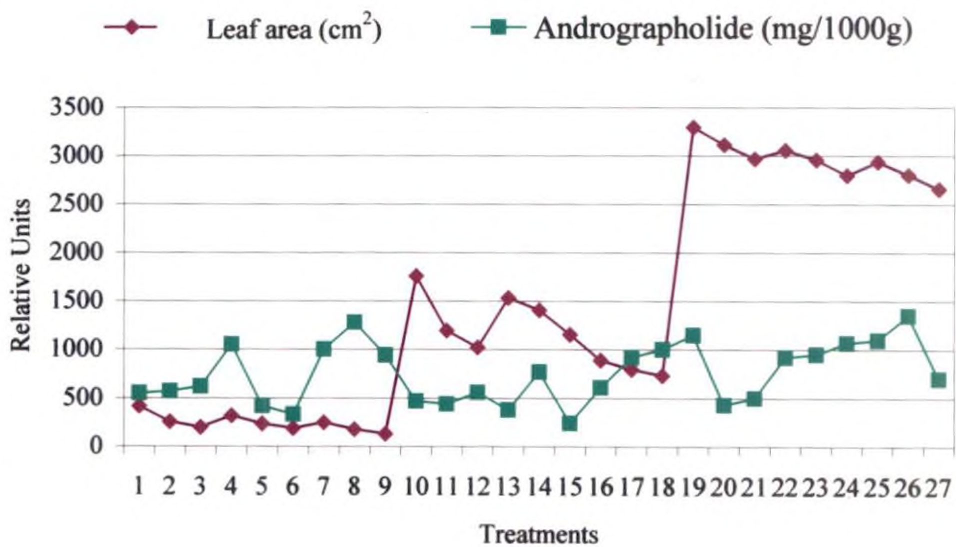


Fig. 7. Relationship between the leaf area and andrographolide content in open condition

50% shade and 75% shade are given in Fig 4, 5, 6 respectively. There was only a week correlation between andrographolide content and plant biomass.

4.1.3 Leaf Area (cm^2)

Significant variation was observed in the total leaf area of the plants under open and different shade levels. Table 5 shows the data of the leaf area of all stress conditions .In the open conditions, the mean leaf area recorded was 1453.56 cm^2 and the range was from 130 cm^2 to 3294 cm^2. The treatment combinations of OG1S1W1, OG1S1W2, OG1S1W3, OG1S2W1, OG1S2W2, OG1S2W3, OG1S3W1, OG1S3W2 and OG1S3W3 reduced leaf area significantly. The reduction in leaf area was 71.3 to 91 per cent compared to overall mean value. The treatment combinations of OG3S1W1, OG3S1W2, and OG3S1W3, were statistically non-significant. The frequency distribution of treatment combinations indicates that 33.3 per cent of treatment combinations had less than 1302.37 cm^2 and 33.3 per cent of treatment combinations had more than 1604.72 cm^2 (Table 5 a).

In fifty per cent of shade level, the leaf area of plants varied between 380.90 to 6019.80 cm^2 per plant and the mean value of 2289.02 cm^2 per plant. The plant leaf area was reduced significantly by the treatment combinations of FG1S1W1, FG1S1W2, FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG1S3W1, FG1S3W2 and FG1S3W3.The reduction in the leaf area was 87.2 to 99 per cent compared to the mean value. The treatment combinations of FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W3, FG3S3W1, FG3S2W2, FG3S2W3 and FG3S3W1 did not show significant reduction in leaf area. The frequency distribution indicated that 40.7 per cent of treatment combinations had leaf area of less than 2379.85 cm^2 and 37.03 per cent had more than 3275.043 cm^2 (Table 5a).

The mean leaf area recorded was 1853.79 cm^2 and the range in leaf area was from 316.81 to 4701.33 cm^2 under seventy five per cent of shade level. The significantly reduced leaf area was observed in treatment combinations of HG1S1W1, HG1S1W2, HG1S1W3, HG1S2W1, HG1S2W2, HG1S2W3, HG1S3W1 and HG1S3W2. These treatment combinations recorded 71 to 99 per

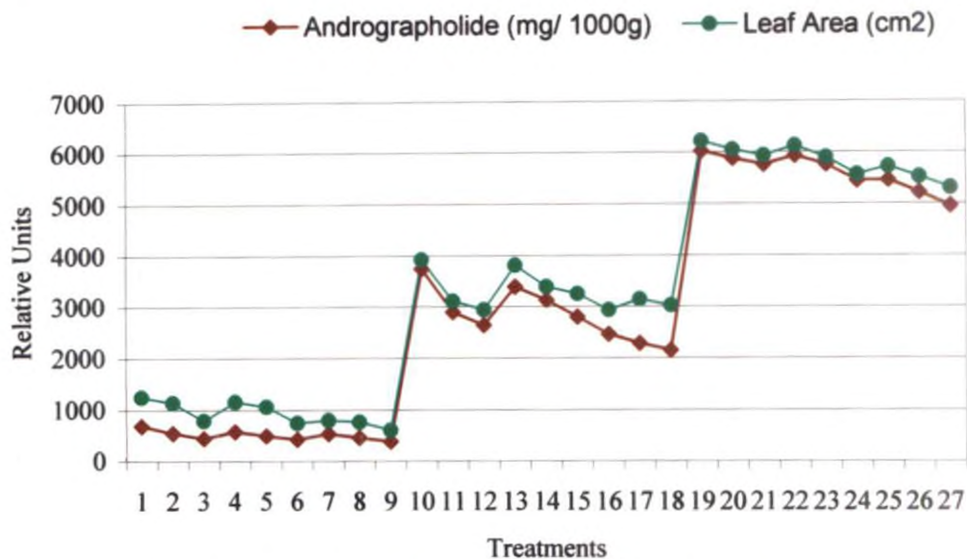


Fig. 8. Relationship between the andrographolide content and leaf area in 50 % shade

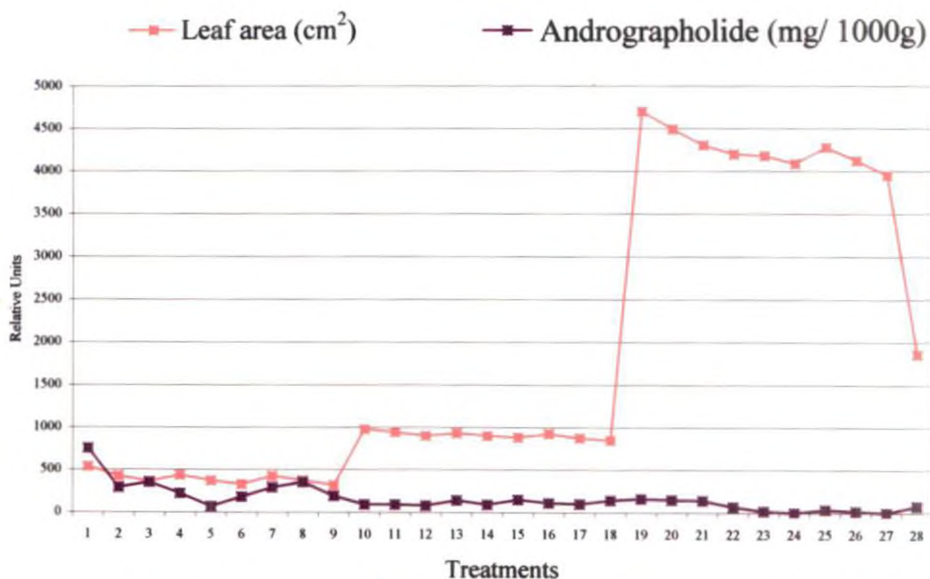


Fig. 9. Relationship between the andrographolide content and Leaf area in 75% shade level

Table 5. Effect of abiotic stress on leaf area of *Andrographis paniculata* under different shade condition

Sl. No	Treatment	Leaf area (cm^2)		
		Open	50% shade	75% shade
1	G1S1W1	416.90	684.68	533.37
2	G1S1 W2	253.62	547.88	424.71
3	G1S1W3	199.24	447.07	361.95
4	G1S2W1	319.39	578.23	434.73
5	G1S2W2	234.43	493.40	371.70
6	G1S2W3	186.25	424.20	329.34
7	G1S3W1	248.31	535.57	421.48
8	G1S3W2	177.15	455.84	373.05
9	G1S3W3	130.10	380.90	316.81
10	G2S1W1	1759.78	3741.91	977.85
11	G2S1W2	1197.10	2891.90	942.69
12	G2S1W3	1023.06	2637.67	899.02
13	G2S2W1	1533.08	3389.94	931.17
14	G2S2W2	1408.17	3126.11	899.34
15	G2S2W3	1156.23	2800.59	879.74
16	G2S3W1	891.38	2459.70	921.42
17	G2S3W2	797.98	2281.71	874.75
18	G2S3W3	734.88	2151.35	845.22
19	G3S1W1	3294.31	6019.80	4701.33
20	G3S1W2	3113.70	5874.27	4493.24
21	G3S1W3	2965.93	5753.34	4304.10
22	G3S2W1	3058.99	5922.66	4197.58
23	G3S2W2	2958.85	5754.11	4180.11
24	G3S2W3	2796.50	5436.01	4087.43
25	G3S3W1	2937.19	5446.12	4277.86
26	G3S3W2	2798.51	5204.39	4121.44
27	G3S3W3	2655.21	4943.37	3950.95
	Mean	**1453.56**	**2977.13**	**1856.79**
	CD (5%)	**303.12**	**597.28**	**413.63**

Table 5a: Grouping of 27 treatments and their frequency distribution for leaf area under different shade condition.

Leaf area under open				Leaf area under fifty per cent Shade			Leaf area under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<1302.37	1,2,3,4,5,6,7 ,8,9,	-9-	<2379.5	1,2,3,4,5,6,7 ,8,9	-11-	1647.48	1,2,3,4,5,6,7,8,9 ,10,11,12,13,14, 15,16,17, 18,19	-19-
Mean ± SE	1302.37- 1604.72	Rest of the treatments	-9-	2379.85- 3275.04	Rest of the treatments	-6-	1647.48- 2060.10	NIL	
Mean+SE	>1604.72	19,20,21, 22,23,24, 25,26,27	-9-	>3275.04	10,19,20,21, 22,23,24,25, 26,27	-10-	2060.10	20,21,22,23, 24,25,26,27	-8-

cent reduction in leaf area compared to mean value. The treatment combinations of HG3S1W1, HG3S1W2 and HG3S1W3, were statistically non-significant. The frequency distribution of treatment combinations indicates that 70.37 per cent had of them less than 1647.48 cm^2 and 33.3 per cent of treatment combination had more than 2060.1 cm^2 (Table 5a). The relationship between andrographolide content and total leaf area of plants grown under open, 50% and 75% shade are given in the Fig 7, 8, and 9of respectively. There was a direct relationship between total leaf area between andrographolide content and the relation was stronger at 50% shade.

4.1.4 Leaf Area Duration (LAD)

The leaf area duration showed significant variation among the treatment combination under open and different shade levels (Table.6). In open condition, the leaf area duration of plants varied from 6.00 to 150.81 cm^2 /day with the mean value of 65.08 cm^2 /day. The LAD was reduced significantly by the treatment combinations of OG1S1W1, OG1S1W2, OG1S1W3, OG1S2W1, OG1S2W2, OG1S2W3, OG1S3W1, OG1S3W2 and OG1S3W3 had reduced leaf area significantly. The reduction of leaf area was 74.3 to 90.7 per cent compared to mean value. The treatment combinations of OG3S1W1, OG3S1W2, and OG3S2W1 were statistically non-significant. The frequency distribution of treatment combinations indicated that 59 per cent of treatment combinations had LAD of less than 58.5 cm^2/day and 33.3 per cent of had more than 71.91 cm^2/day (Table 6 a).

In fifty per cent of shade level, the overall mean and range were 18.58 cm^2 /day and 274.50 to 110.94 cm^2 /day respectively. The LAD was reduced significantly by the treatment combinations of FG1S1W1, FG1S1W2, FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG1S3W1, FG1S3W2 and FG1S3W3. The reduction of leaf area duration was 83.25 to90.7 per cent compared to the mean value. The treatment combinations of FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W3 and FG3S3W1 were statistically non significant. The frequency distribution of treatment combinations indicates that 62.91 per cent had

Table 6. Effect of abiotic stress on leaf area duration of *Andrographis paniculata* under different shade condition (cm^2/day).

Sl. No	Treatment	Leaf area duration		
		Open	50% shade	75% shade
1	G1S1W1	16.68	30.49	24.48
2	G1S1 W2	10.99	25.74	20.78
3	G1S1W3	8.87	21.36	18.08
4	G1S2W1	13.82	27.14	21.25
5	G1S2W2	10.62	23.82	18.80
6	G1S2W3	8.70	20.84	17.03
7	G1S3W1	10.92	25.40	20.87
8	G1S3W2	7.90	21.81	18.66
9	G1S3W3	6.00	18.58	16.24
10	G2S1W1	66.93	128.39	58.39
11	G2S1W2	50.95	105.26	56.29
12	G2S1W3	45.76	98.30	53.68
13	G2S2W1	60.16	118.34	55.60
14	G2S2W2	56.62	110.55	53.70
15	G2S2W3	49.21	101.93	52.53
16	G2S3W1	41.69	93.53	55.02
17	G2S3W2	38.54	87.97	52.23
18	G2S3W3	36.10	83.53	50.47
19	G3S1W1	150.81	274.50	214.38
20	G3S1W2	142.55	267.86	204.89
21	G3S1W3	135.78	262.35	196.26
22	G3S2W1	140.04	270.07	191.41
23	G3S2W2	135.46	262.38	190.61
24	G3S2W3	128.02	247.88	186.38
25	G3S3W1	134.47	248.34	195.07
26	G3S3W2	128.12	237.32	187.93
27	G3S3W3	121.56	225.41	180.16
	Mean	**65.08**	**110.94**	**75.35**
	CD (5%)	**13.702**	**26.17**	**19.16**

Table 6a: Grouping of 27 treatments and their frequency distribution for leaf area duration under different shade condition.

Leaf area duration under open				Leaf area duration under fifty per cent shade			Leaf area duration under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<58.25	1,2,3,4,5,6,7,8,9,10, 11,12,13,14, 15,16,17,18,	-18-	<114.32	1,2,3,4,5,6,7,8,9,10,11,12,14,15,16,17,18,	-17-	<79.8	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17, 18,	-18-
Mean ± SE	58.28-71.91	NIL	-0-	114.32-140.42	Rest of the Treatments	-1-	79.8-98.8	NIL	-0-
Mean+SE	>71.91	19,20,21,22, 23,24,25,26, 27	-9-	>140.42	19,20,21,22,23,24,25,26,27,	-9-	>98.8	19,20,21,22, 23,24,25,26, 27	-9-

less value than 114.32 cm^2 /day and 33.3 per cent had more than140.42 cm^2 /day (Table 6 a).

The mean leaf area duration of seventy five per cent of shade level was 75.35 cm^2 /day and range in leaf area was from 16.24 to 214.38 cm^2 /day. The leaf area duration was reduced significantly by the treatment combinations of HG1S1W1, HG1S1W2, HG1S1W3, HG1S2W1, HG1S2W2, H1S2W3, H1S3W1, H1S3W2 and HG1S3W3. The reduction of leaf area duration was 72.5 to 81.8 per cent compared to mean value. The treatment combinations of HG3S1W1, HG3S1W2, HG3S1W3 were statistically non-significant .The frequency distribution of treatment combinations indicates that 66.6 per cent of treatment combinations had LAD of less than 79.8 cm^2 /day and 33.3 per cent had more than 98.8 cm^2/day (Table 6 a).

4.1.5 Specific Leaf Area (cm^2/g)

Specific leaf area showed significant variation among the treatment combinations under open and different shade levels Table 7 shows the SLA under different shade and open levels. In open condition, the mean specific leaf area was 3.97 cm^2 /g and the range was 4.61 to 44.40 cm^2 /g. The significant treatment combinations were OG2S2W1, G2S2W2, OG2S2W3, OG2S3W1, OG2S3W2, OG2S3W3, OG3S1W1, OG3S1W2, OG3S1W3, OG3S2W1, OG3S2W2, OG3S2W3, OG3S3W1, OG3S3W2, and OG3S3W3 where these treatments had reduced SPA. The per cent of reduction was 53.13 to 74.61 per cent compared to the mean value. The treatment combinations of OG1S1W1, OG1S1 W2, OG1S1W3, and OG1S2W1 had no any influence on relative growth rate. The frequency distribution indicated that 66.6 per cent of treatment combinations had SPA of less than 16.17 cm^2 /g and 33.3 per cent had more than 20.14 cm^2 /g (Table 7 a)

In fifty per cent shade level, the mean specific leaf area was 79.74 cm^2 /g and range specific leaf area was 69.12 to 95.46 cm^2 /g. The treatment combinations of FG1S1W1, FG1S1W2, FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG1S3W1, FG1S3W2, FG1S3W3, FG2S1W1, FG2S1W2,

Table 7. Effect of abiotic stress on Specific Leaf Area of *Andrographis paniculata* under different shade condition (cm^2/g).

Sl. No.	Treatment	Specific leaf area		
		Open	50% shade	75% shade
1	G1S1W1	44.40	74.71	72.57
2	G1S1 W2	42.28	76.70	71.67
3	G1S1W3	41.90	77.50	70.59
4	G1S2W1	44.02	77.47	71.84
5	G1S2W2	39.86	78.31	76.29
6	G1S2W3	38.04	79.13	77.37
7	G1S3W1	39.99	78.56	78.98
8	G1S3W2	39.17	79.23	78.93
9	G1S3W3	37.50	78.35	78.72
10	G2S1W1	9.14	68.05	72.42
11	G2S1W2	9.05	69.50	73.42
12	G2S1W3	8.83	69.98	73.02
13	G2S2W1	8.51	68.16	69.50
14	G2S2W2	8.49	69.23	70.53
15	G2S2W3	8.35	69.71	73.75
16	G2S3W1	8.84	68.69	70.77
17	G2S3W2	8.25	69.13	72.63
18	G2S3W3	8.11	69.12	70.74
19	G3S1W1	5.73	88.33	55.05
20	G3S1W2	5.58	89.73	54.80
21	G3S1W3	5.39	90.55	54.95
22	G3S2W1	5.05	92.02	55.07
23	G3S2W2	4.95	91.93	54.94
24	G3S2W3	4.80	94.13	54.56
25	G3S3W1	4.85	94.58	54.34
26	G3S3W2	4.74	95.46	54.14
27	G3S3W3	4.61	94.81	52.71
	Mean	**18.16**	**79.74**	**67.20**
	CD (5%)	**3.97**	**13.15**	**11.10**

Table 7a: Grouping of 27 treatments and their frequency distribution for specific leaf area under different shade condition.

Specific leaf area under open				Specific leaf area under fifty per cent shade			Specific leaf area under seventy five per cent Shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<16.17	10,11,12, 13,14,15, 16,17,18,19, 20,21,22,23, 24,25,26,27	-18-	73.18	10,11,12,13,1 4,15,16,17,18	-9-	<61.66	19.20.21,22,2 3,24,25,26,27	-9-
Mean ± SE	16.17- 20.1433	NIL	-0-	73.18- 86.26	Rest of the Treatments	-9-	91.66	Rest of the Treatments	-6-
Mean+SE	>20.14	1,2,3,4,5,6,7 ,8,9,	-9-	86.26	19,20,21,22,2 3,24,25,26,27	-9-	>72.72	1,2,3,4,5,6,7,8 ,9,10,11,12,	-12-

FG2S1W3, FG2S2W1, FG2S2W2, FG2S2W3, FG2S3W1, FG2S3W2, FG2S3W3 and FG3S1W1 showed significant reduction in SLA compared to mean value. The treatment combinations of FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W2, FG3S2W3, FG3S3W1, FG3S3W2 and FG3S3W3 were statistically non significant. The reduction in SPA was 50 to 67 per cent compared to mean value. The frequency distribution indicated that 33.3 per cent of treatment combinations had SPA of less than 73.18 cm^2/g and 33.3 per cent had more than 86.26 cm^2/g (Table 7 a).

In seventy five per cent of shade, the overall mean was 11.10 cm2 /g and range was 52.71to 78.72 cm^2/g respectively. The SPA was reduced significantly in the treatment combinations of HG3S1W1, HG3S1W2, HG3S1W3, HG3S2W1, HG3S2W2, HG3S2W3, HG3S3W1, HG3S3W2 and HG3S3W3. The per cent of reduction was 18.08 to 21.55 per cent compared to mean value. The treatment combination of HG1S1W1, HG1S1W2, HG1S1W3, HG1S2W1, HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2, HG1S3W3, HG2S1W1, HG2S1W2, HG2S1W3, HG2S2W1, HG2S2W2, HG2S2W3, HG2S3W1, HG2S3W2, HG2S3W3 and HG3S1W1 were statistically non significant. The frequency distribution indicated that 33.3 per cent of treatment combinations had SLA of less than 61.66 cm^2/g and 44 per cent had more than 72.72 cm^2/g (Table 7 a)

4.1.6 Relative growth rate (mg g^{-1} day^{-1})

Relative growth rate showed significant variation among the treatment combinations under open and different shade levels (Table 8). In open condition, the mean relative growth rate was 19.9 mg g^{-1} day^{-1} and the range was from 0.8 to 52.6 mg g^{-1} day^{-1}. The significant treatments OG2S1W1, OG2S2W1 had reduced RGR and the per cent of reduction ranged from 75 to 95 compared to the mean value. The treatment combinations of OG3S1W1, OG3S1W2, and OG3S2W1 had no any influence on relative growth rate. The frequency distribution of treatment combinations reveals that 3 per cent of treatment combination had RGR of less than 17.7 mg g^{-1} day^{-1} and 3 per cent had more than 21.19 mg g^{-1} day^{-1} (Table 8 a).

Table 8. Effect of abiotic stress on Relative Growth Rate of *Andrographis paniculata* under different shade condition (mg g^{-1} day^{-1}).

Sl. No	Treatment	Relative growth rate		
		Open	50% shade	75% shade
1	G1S1W1	20.4	34.2	27
2	G1S1 W2	14.1	26.6	21
3	G1S1W3	12.1	24.7	19.5
4	G1S2W1	14.2	26.6	21
5	G1S2W2	10.9	22.8	18
6	G1S2W3	8.8	19	15
7	G1S3W1	13	24.7	19.5
8	G1S3W2	12	24.7	19.5
9	G1S3W3	9.6	20.9	16.5
10	G2S1W1	52.6	41.8	33
11	G2S1W2	43.4	36.1	28.5
12	G2S1W3	39.7	30.4	24
13	G2S2W1	49.9	43.7	34.5
14	G2S2W2	47.9	41.8	33
15	G2S2W3	43.4	36.1	28.5
16	G2S3W1	36.6	26.6	21
17	G2S3W2	34.5	24.7	19.5
18	G3S3W3	33.4	22.8	18
19	G3S1W1	7.2	13.3	10.5
20	G3S1W2	6	13.3	10.5
21	G3S1W3	4.9	11.4	9
22	G3S2W1	6	13.3	10.5
23	G3S2W2	4.9	11.4	9
24	G3S2W3	2.4	9.5	7.5
25	G3S3W1	4.3	11.4	9
26	G3S3W2	2.5	9.5	7.5
27	G3S3W3	0.8	7.6	6
	Mean	**19.83**	**23.29**	**18.38**
	CD (5%)	**4.26**	**4.18**	**3.30**

Table 8a: Grouping of 27 treatments and their frequency distribution for relative growth rate under different shade condition.

Relative growth rate under open				Relative growth rate under fifty per cent shade			Relative growth rate under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<17.70	27	-1-	<21.20	6,9,19,20,21, 22,23,24,25,26,27,	-11-	>16.73	19,20,21,22, 23,24,25,26,27,	-9-
Mean ± SE	17.70-21.95	Rest of treatments	-17-	21.20-25.37	Rest of the treatments	-7-	16.73-20.02	Rest of the treatments	-10-
Mean+SE	>21.95	10,11,12, 13,14,15,16,17,18,	-9-	>25.37	1,4,10,11,12, 13,14,15,16,	-9-	<20.02	1,2,11,12,13, 14,15,16,	-8-

In fifty per cent shade level, the mean relative growth rate was 23.29 mg g^{-1} day^{-1} and range was from 7.6 to 41.8 mg g^{-1} day^{-1}. The treatment combinations of FG3S1W3, FG3S2W1, FG3S2W2, FG3S2W3, FG3S3W1, FG3S3W2, and FG3S3W3 showed significant reduction in RGR compared to mean value. The treatment combinations of FG2S1W1, FG2S2W1 and FG2S2W2 were statistically non significant. The reduction in RGR was 42.89 to 67.6 per cent compared to mean value. The frequency distribution of treatment combination indicates that 48.18 per cent of treatment combinations had RGR of less than 11.4 and 25.9 per cent had more than 13.8 mg g^{-1} day^{-} (Table 8 a).

Under seventy five per cent shade, the overall mean and range of RGR were 18.38 mg g^{-1} day^{-1} and 6 to 28.5 mg g^{-1} day^{-1} respectively. The RGR was reduced significantly by the treatment combinations of HG3S1W3, HG3S2W1, HG3S2W2, HG3S2W3, HG3S3W1, HG3S3W2, and HG3S3W3. The reduction was 42.4 to 67.36 per cent compared to mean value. The treatment combination of HG1S1W3, HG1S2W2, HG1S1W3, HG2S1W1, HG2S2W1 and HG2S2W2 were statistically non significant. The frequency distribution indicates that 3.7 per cent had RGR of less than 11 mg g^{-1} day^{-1} and 37 per cent of treatment combinations had more than 13.3 mg g^{-1} day^{-1} (Table 8a).

4.1.7 Net Assimilation Rate (mg cm^{-2} day^{-1})

The net assimilation rate showed significant variation under different shade and open conditions (Table 9). In open condition, the mean NAR was 1.7845 mg cm^{-2} day^{-1} and the range was from 0.07 to 4.41 mg cm^{-2} day^{-1}. The net assimilation rate was significantly reduced in the treatment combinations of OGS3W1, OG3S1W2, OG3S2W3, OGS2W1, OG3S2W3, OGS3W1, OG3S3W2 and OGS3W3. The reduction of NAR was 40 to 96 percent compared to the mean value. The statistically non significant treatment combinations were OG2S1W1, OG2S2W1 and OG2S2W2. The frequency distribution of treatment combinations indicates that 62.9 per cent of treatment combinations had NAR of less than 1.5927 mg cm^{-2} day^{-1} and 33 per cent had more than 1.9775 mg cm^{-2} day^{-1} (Table 9a).

Table 9. Effect of abiotic stress on Net Assimilation Rate of *Andrographis paniculata* under different shade condition (mg cm^{-2} day^{-1}).

Sl. No.	Treatment	Net assimilation rate		
		Open	50% shade	75% shade
1	G1S1W1	1.57	3.95	2.52
2	G1S1 W2	1.29	2.86	1.99
3	G1S1W3	1.20	2.59	1.83
4	G1S2W1	1.21	2.87	2.00
5	G1S2W2	1.00	2.41	1.72
6	G1S2W3	0.83	2.11	1.55
7	G1S3W1	1.25	2.78	1.89
8	G1S3W2	1.21	2.64	1.82
9	G1S3W3	1.05	2.28	1.64
10	G2S1W1	4.41	4.36	2.30
11	G2S1W2	3.85	3.79	1.79
12	G2S1W3	3.64	3.19	1.64
13	G2S2W1	4.27	4.72	2.21
14	G2S2W2	4.30	4.36	2.12
15	G2S2W3	3.99	3.55	1.87
16	G2S3W1	3.53	2.43	1.44
17	G2S3W2	3.35	2.19	1.33
18	G3S3W3	3.36	2.07	1.28
19	G3S1W1	0.50	1.32	0.90
20	G3S1W2	0.43	1.19	0.85
21	G3S1W3	0.36	1.08	0.78
22	G3S2W1	0.42	1.19	0.87
23	G3S2W2	0.36	1.08	0.81
24	G3S2W3	0.19	0.85	0.64
25	G3S3W1	0.33	1.05	0.76
26	G3S3W2	0.20	0.89	0.65
27	G3S3W3	0.07	0.725	0.54
	Mean	**1.78**	**2.38**	**1.47**
	CD (5%)	**0.38**	**0.43**	**0.25**

Table 9a: Grouping of 27 treatments and their frequency distribution for net assimilation rate under different shade condition.

	Net assimilation rate under open			Net assimilation rate under fifty per cent shade			Net assimilation rate under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<1.59	2,3,4,5,6,7, 8,9,19,20,21,2 2,23, 24,25,26, 27	-17-	<2.17	6,18,19,21,22, 23,24,25,26, 27	-9-	<1.34	1,17,18,19,20, ,22,23,24,25, 26,27	-11-
Mean $\pm$ SE	1.59- 1.97	Rest of the treatments	-1-	2.17- 2.60	Rest of treatments	-5-	1.34- 1.60	Rest of treatments	-2-
Mean+SE	>1.97	10,11,12, 13,14,15, 16,17,18	-9-	>2.60	1,2,4,7,8,10, 11,12,13,14, 15,	-12-	>1.60	1,2,3,4,5,7,8,9 ,10,11,12,13,4 ,15,16	-14-

In fifty per cent shade level, the NAR of plants varied from 0.72 to 4.72 mg cm^2 day^{-1} with the mean value of 2.38 mg cm^{-2} day^{-1} was observed. The significant reduction of NAR was observed in treatment combinations of FG3S1W3, FG3S2W2, FG3S2W3, FG3S3W1, FG3S3W2 and FG3S3W3. The per cent of reduction was 22.9 to 68.34 compared to mean value. The treatment combinations of FG2S1W1, FG2S2W1 and FG2S2W2 were statistically non significant. The frequency distribution of treatment combinations indicates that 33.3 per cent had less than 2.71 mg cm^{-2} day^{-1} and 44.4 per cent had more than 2.60 mg cm^{-2} day^{-1} (Table 9 a)

In seventy five per cent shade level, the overall mean and range of NAR were 1.47 and 0.54 to 2 mg cm^{-2} day^{-1} respectively. The NAR was reduced significantly by the treatment combinations of HG3S1W3, HG3S2W3, and HG3S3W2 and HG3S3W3.The reduction of NAR was 43 to 47 per cent compared to mean value. The treatment combinations of HG2S1W1and HG1S1W1 had no significant change due to stress. The frequency distribution of treatment combinations indicates that 40.74 per cent of treatment combinations had NAR of less than 1.34 mg cm^{-2} day^{-1} and 51.85 per cent of treatment combinations had more than 1.60 mg cm^{-2} day^{-1} (Table 9 a)

4.2. PHYSIOLOGICAL PARAMETERS

4.2.1 Stomatal Conductance (cms^{-1})

Stomatal conductance showed significant variation among the treatment combinations under open and different shade levels (Table.10). In open condition, the mean stomatal conductance was 0.157 cms^{-1} and the range was from 0.020 to 0.270. The significant treatment combinations were OG3S1W2, G3S1W3, G3S2W1, G3S2W3, G3S3W1, G3S3W2 and G3S3W3 where these treatments recorded reduced stomatal conductance. The per cent of reduction was 12.3 to 89.4 per cent compared to mean value. The treatment combination of was OG1S1W1, OG1S1W2, were statistically non- significant. The frequency distribution indicated that 40.7 per cent of treatment combinations had stomatal

Table 10. Effect of abiotic stress on Stomatal Conductance of *Andrographis paniculata* under different shade condition

Sl. No	Treatment	Stomatal conductance cm/s		
		Open	50% shade	75% shade
1	G1S1W1	0.286	0.076	0.222
2	G1S1 W2	0.270	0.074	0.211
3	G1S1W3	0.256	0.061	0.190
4	G1S2W1	0.250	0.072	0.173
5	G1S2W2	0.139	0.063	0.179
6	G1S2W3	0.238	0.069	0.128
7	G1S3W1	0.231	0.070	0.154
8	G1S3W2	0.227	0.120	0.147
9	G1S3W3	0.179	0.068	0.143
10	G2S1W1	0.233	0.114	0.056
11	G2S1W2	0.208	0.159	0.055
12	G2S1W3	0.204	0.105	0.052
13	G2S2W1	0.192	0.112	0.035
14	G2S2W2	0.192	0.107	0.052
15	G2S2W3	0.189	0.105	0.047
16	G2S3W1	0.190	0.094	0.045
17	G2S3W2	0.120	0.104	0.038
18	G2S3W3	0.183	0.101	0.037
19	G3S1W1	0.025	0.045	0.260
20	G3S1W2	0.024	0.044	0.154
21	G3S1W3	0.020	0.043	0.219
22	G3S2W1	0.023	0.042	0.112
23	G3S2W2	0.022	0.035	0.214
24	G3S2W3	0.021	0.038	0.179
25	G3S3W1	0.018	0.049	0.206
26	G3S3W2	0.021	0.037	0.199
27	G3S3W3	0.020	0.036	0.191
	Mean	**0.14**	**0.07**	**0.13**
	CD (5%)	**0.02**	**0.01**	**0.02**

Table 10a. Grouping of 27 treatments and their frequency distribution for Stomatal conductance under different shade condition.

Stomatal conductance under open				Stomatal conductance under fifty per cent shade			Stomatal conductance under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<0.11	17,18,19,20, 21,22,23,24, 25,26,27	-11-	>0.06	3,5,9,19,20,21,2 2,23,24,25, 26,27	-12-	<0.12	10,11,12,13, 14,15,16,17, 18,19,	-10-
Mean ± SE	0.11- 0.17	Rest of the treatments	-1-	0.068- 0.08	Rest of the treatments	-5-	0.12- 0.14	Rest of the treatments	-1-
Mean+SE	>0.17	1,2,3,4,6,7,8 ,9,10,11,12, 13,14,15,16,	-15-	<0.08	8,10,11,12,13,1 4,15,16,17, 18	-10-	>0.14	1,2,3,4,5,6,7,8 ,19,20,21,23, 24,25,26,27	-16-

conductance less than 0.11 cms^{-1} and 55.5 per cent had more than 0.17 cms-1 (Table.10a).

In fifty per cent shade level, the mean stomatal conductance was 0.076 cms-1 and range stomatal conductance was 0.036 to 0.159 cms^{-1} The treatment combinations of FG3S1W1, FG3S1W2, FG3S1W3, FG3S2W1, FG3S2W2, FG3S2W3, FG3S3W1, FG3S3W2, and FG3S3W3 showed significant reduction in stomatal conductance compared to mean value. The per cent of reduction was 40.78 to 52.6 per cent compared to mean value. The treatment combination of FG2S1W1, was statistically non significant. The frequency distribution indicated that 44.4 per cent of treatment combinations had value less than 0.0686 cms^{-1} and 37.03 per cent had more than 59.25 (Table.10a).

In seventy five per cent of shade, the overall mean and range were 0.137 cms^{-1} and 0.037 to 0.055 cms^{-1} respectively. The stomatal conductance was reduced significantly by the treatment combinations of HG2S1W1, HG2S1W2, HG2S2W1, HG2S2W1, HG2S2W2, HG2S2W3, HG2S3W1, HG2S3W2 and HG2S3W3. The per cent of reduction was 59.12 to 72.9 per cent compared to mean value. The treatment combinations of HG3S1W1, and HG3S1W2 were statistically non significant. The frequency distribution indicated that 37.03 per cent of treatment combinations had value less than 0.1241 cms^{-1} and 59.25 per cent had more than 0.1499 cms^{-1} (Table.10a).

4.2.2 *Stomatal frequency*

Stomatal frequency showed significant variation among the treatment combinations under open and different shade levels (Table.11). In open condition, the mean stomatal frequency was 815.92 and the range was from 612 to 1024. The significant treatment combinations were OG2S3W1 OG2S3W2, OG2S3W3, OG3S1W1, OG3S1W2, OG3S1W2, OG3S1W3, OG3S2W1, OG3S2W2, OG3S2W3, OG3S3W1, OG3S3W2 and OG3S3W3 here these treatments recorded reduced stomatal frequency. The per cent of reduction was 15.36 to 24.9 per cent compared to mean value. The treatment combination of was OG1S1W1, OG1S1W2, OG1S1W3, OG1S2W1, OG1S2W2, OG1S2W3, OG1S3W1,

Table 11. Effect of abiotic stress on Stomatal frequency of *Andrographis paniculata* under different condition

Sl. No	Treatment	Stomatal frequency (no/cm^2)		
		Open	50% shade	75% shade
1	G1S1W1	1012	689	920
2	G1S1 W2	1001	612	910
3	G1S1W3	986	596	896
4	G1S2W1	1024	512	930
5	G1S2W2	895	502	813
6	G1S2W3	956	589	869
7	G1S3W1	986	621	896
8	G1S3W2	853	608	775
9	G1S3W3	753	624	542
10	G2S1W1	895	654	896
11	G2S1W2	985	645	853
12	G2S1W3	845	632	754
13	G2S2W1	988	624	735
14	G2S2W2	877	618	725
15	G2S2W3	758	634	712
16	G2S3W1	712	612	732
17	G2S3W2	702	628	705
18	G2S3W3	712	658	695
19	G3S1W1	693	689	654
20	G3S1W2	689	695	635
21	G3S1W3	652	569	625
22	G3S2W1	682	542	654
23	G3S2W2	642	523	687
24	G3S2W3	612	502	635
25	G3S3W1	721	542	625
26	G3S3W2	704	563	612
27	G3S3W3	695	512	635
	Mean	**815.92**	**599.81**	**745.18**
	CD (5%)	**135.39**	**98.63**	**123.37**

Table 11a. Grouping of 27 treatments and their frequency distribution for Stomatal frequency under different shade condition.

Stomatal frequency under open				Stomatal frequency under fifty percent shade			Stomatal frequency under seventy five percent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<748.39	16,17,18, 19,20,21,22,23,24,25,26,27	-12-	<550.64	4,5,22,23,24, 25,27,	-7-	<683.644	19,20,21,22, 24,25,26,27	-8-
Mean ± SE	748.39-883.45	Rest of the Treatments	-2-	550.64-649	Rest of the Treatments	-17-	683.644-806.71	Rest of the Treatments	-11-
Mean+SE	>883.45	1,2,3,4,5,6,7, 8,10,11,12,13, 14	-13-	>649	1,18,19	-3-	>806.71	1,2,3,4,5,6,7, 10,	-8-

OG2S1W1 OG2S2W1 and OG2S1W2 were statistically non- significant. The frequency distribution indicated that 44 per cent of treatment combinations had stomatal frequency less than 748.39 and 48 per cent had more than 883.45 (Table.11a).

In fifty per cent shade level, the mean stomatal frequency was 599.81and range stomatal conductance was 502 to 695. The treatment combinations of FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG3S1W3, FG3S2W1, FG3S2W2, FG3S2W3, FG3S3W1, FG3S3W2, and FG3S3W3 showed significant reduction in stomatal conductance compared to mean value. The per cent of reduction was 5 to 16.36 per cent compared to mean value. The treatment combination of FG1S1W1, FG1S1W2, FG1S3W2, FG1S3W3, FG2S1W1, FG2S1W2, FG2S1W3, FG2S2W1, FG2S2W2, FG2S2W3, FG2S3W1, FG2S3W2, FG2S3W3, FG3S1W1, FG3S1W2 was statistically non significant. The frequency distribution indicated that 25 per cent of treatment combinations had value less than 550.644 and 11.1 per cent had more than 649 (Table.11a).

In seventy five per cent of shade, the overall mean and range were 745.18 and 542 to 920 respectively. The stomatal frequency was reduced significantly by the treatment combinations of HG1S1W1, HG1S1W2, HG1S1W3, HG1S2W2, HG1S2W3, HG1S2W3, HG1S3W1, HG1S3W2, HG1S3W3, HG2S1W1 and HG2S1W2. The per cent of reduction was 12.6 to27.24 per cent compared to mean value. The treatment combinations of HG1S3W3, HG3S1W1, HG3S1W2, HG3S1W3, HG3S2W1, HG3S2W2, HG3S2W3, HG3S3W1, HG3S3W2, HG3S3W3 were statistically non significant. The frequency distribution indicated that 29.62 per cent of treatment combinations had value less than 683.644 and 29.2 per cent had more than 806.716 respectively (Table.11a)

4.2.3 Chlorophyll Content (mg g^{-1} fresh leaf)

The chlorophyll content showed significant variation under different shade levels and open condition Table 12 shows the data of chlorophyll 'a' content plants grown under of all shade and open conditions. In open condition, the mean chlorophyll 'a' content was 1.02 mg g^{-1} fresh leaf and with a range from 0.43 to

Table 12. Effect of abiotic stress on Chlorophyll content of *Andrographis paniculata* under different shade condition

Sl. No	Treatment	Chlorophyll A			Chlorophyll b			Total chlorophyll		
		Open	50%	75%	open	50%	75%	Open	50%	75%
1	G1S1W1	0.679	1.542	0.788	0.285	0.731	0.311	0.963	2.273	1.099
2	G1S1 W2	1.101	1.536	1.538	0.243	0.578	0.665	1.344	2.113	2.202
3	G1S1W3	1.061	1.573	0.987	0.361	0.990	0.365	1.422	2.562	1.351
4	G1S2W1	0.433	1.553	1.454	0.077	0.850	0.411	0.510	2.402	1.864
5	G1S2W2	0.912	1.473	1.494	0.369	0.548	0.129	1.281	2.020	1.622
6	G1S2W3	1.092	1.588	1.444	0.667	0.960	0.737	1.758	2.548	2.181
7	G1S3W1	0.751	1.593	1.398	0.266	1.129	0.512	1.016	2.721	1.910
8	G1S3W2	0.893	1.562	1.558	0.313	1.082	0.755	1.205	2.643	2.312
9	G1S3W3	0.919	1.524	0.540	0.223	1.297	0.117	1.142	2.821	0.656
10	G2S1W1	1.455	1.281	1.567	0.498	0.525	1.093	1.953	1.805	2.659
11	G2S1W2	1.559	1.332	1.411	0.534	0.574	0.379	2.093	1.906	1.790
12	G2S1W3	1.400	1.175	1.695	0.404	0.381	0.286	1.804	1.556	1.980
13	G2S2W1	1.487	1.081	0.775	0.426	0.407	0.131	1.912	1.487	0.906
14	G2S2W2	0.754	1.375	1.526	0.340	0.500	0.872	1.094	1.875	2.397
15	G2S2W3	0.873	1.326	1.019	0.499	0.543	0.486	1.371	1.869	1.505
16	G2S3W1	1.373	1.316	1.324	0.442	0.654	0.455	1.814	1.970	1.779
17	G2S3W2	1.209	1.332	1.345	0.343	0.523	0.373	1.552	1.855	1.718
18	G2S3W3	0.914	1.324	1.255	0.151	0.432	0.360	1.065	1.756	1.615
19	G3S1W1	1.401	0.860	1.352	0.484	0.361	0.439	1.884	1.221	1.791
20	G3S1W2	0.762	1.402	0.872	0.212	0.451	0.450	0.974	1.852	1.321
21	G3S1W3	0.850	1.301	0.980	0.321	0.448	0.245	1.171	1.749	1.224
22	G3S2W1	0.553	1.098	1.522	0.145	0.268	0.698	0.697	1.366	2.219
23	G3S2W2	0.864	1.490	0.888	0.417	0.656	0.247	1.280	2.146	1.135
24	G3S2W3	1.412	1.545	1.300	0.804	1.067	0.553	2.216	2.612	1.852
25	G3S3W1	0.959	1.245	1.434	0.373	0.281	0.828	1.331	1.525	2.261
26	G3S3W2	1.509	1.309	1.065	0.651	0.490	0.415	2.160	1.799	1.479
27	G3S3W3	0.444	1.197	1.043	0.110	0.501	0.272	0.554	1.697	1.314
	Mean	**1.023**	**1.368**	**1.243**	**0.369**	**0.638**	**0.466**	**1.391**	**2.005**	**1.709**
	C.D.(5%)	**0.1756**	**0.2268**	**0.2091**	**0.0662**	**0.1142**	**0.4665**	**0.2413**	**0.3353**	**0.2914**

1.559 mg g^{-1} fresh leaf. The reduction of chlorophyll content was 45.6to 56.21 per cent compared to mean value. The Chlorophyll 'a' content was reduced significantly by the treatment combinations of G3S3W3OG1S2W1, OG3S2W1 and OG3S3W3. The treatment combination of OG2S1W1, OG2S1W2, OG2S1W3, OG2S2W1, OG3S1W1, OG3S2W3 and OG3S3W2 had no influence on chlorophyll content. The frequency distribution of treatment combinations reveals that 48.1 per cent had value less than 0.9352 mg g^{-1} fresh leaf and 33.3 per cent had more than 1.1104 mg g^{-1} fresh leaf (Table 12a).

In fifty per cent of shade condition, the overall mean and range were 1.368 and 0.860 to 1.593 mg g^{-1} fresh leaf respectively. The chlorophyll content was reduced significantly by the treatment combinations of G2S2W1, and G3S1W1. The reduction of chlorophyll content was 20.97 to 37.13 per cent compared to mean value. The treatment combinations of FG1S1W1, FG1S1 W2, FG1S1W3, FG1S2W1, FG1S2W2, FG1S2W3, FG1S3W1, FG1S3W2, FG1S3W3, FG2S2W2, FG3S1W2, FG3S2W2, and FG3S2W3did not show any influence on chlorophyll 'a'. The frequency distribution of treatment combination indicates that 0.3 per cent of treatment combinations had value less than 1.2553 mg g^{-1} fresh leaf and 37.03 per cent had more than 1.4815 mg g^{-1} fresh leaf (Table 12a)

Under seventy five per cent shade level, the chlorophyll 'a' content of the plants varied between 0.54 to 1.558 mg g^{-1} fresh leaves with a mean of 1.24 mg g^{-1} fresh leaf. The treatment combinations of HG1S2W2, HG2S2W3 and HG2S2W2 were recorded significant reduction in chlorophyll 'a' content. The per cent of reduction was 56.55 compared to the mean value. The treatment combination of HS2S1W1 was statistically non-significant. The frequency distribution of treatment combinations reveals that 29 per cent of treatment combinations had value less than 1.139 mg g^{-1} fresh leaf and 51.8 per cent had more than 1.348 mg g^{-1} fresh leaf (Table 12a)

Significant variation existed for chlorophyll 'b' content in open and different shade levels (Table 12). The data on chlorophyll 'b' content of plants under shade and open levels are shown in Table 9. In open condition, the overall

mean and range were 0.369 mg g^{-1} fresh leaf and 0.070 to 1.29 mg g^{-1} fresh leaf respectively. The treatment combinations of OG1S2W1 were reduced chlorophyll 'b' content significantly. The per cent of reduction was 70.18 to 79.13 compare to the mean value. The treatment combinations of OG3S2W3 was statistically non significant. The frequency distribution of treatment combinations showed that 44 per cent of treatment combinations had value less than 0.3359 mg g^{-1} fresh leaf and 37.03 per cent had more than 0.4019 mg g^{-1} fresh leaf (Table 12b).

In fifty per cent of shade level, the overall mean and range of chlorophyll 'b' content were 0.638 and 0.268 to 1.297 mg g^{-1} fresh leaf respectively. The Significant reduction in chlorophyll 'b' content was shown by that treatment combination of FG2S1W3, FG3S1W1, FG3S2W1, and FG3S2W1. The reduction in chlorophyll 'b' content was 23.19 to 57.99 per cent compared to mean value. The non significant treatment combination was FG3S3W3. The frequency distribution of treatment reveals that 55 per cent of treatment combination had value less than 0.5813 mg g^{-1} fresh leaf and 29 per cent had more than 0.6953 mg g^{-1} fresh leaf (Table 12b).

In seventy five per cent of shade level, the mean chlorophyll 'b' content was 0.466 mg g^{-1} fresh leaf and range in chlorophyll 'b' content was from 0.12 to 1.09 mg g^{-1} fresh leaf. The significant reduction in chlorophyll 'b' content was shown by treatment combinations were HG1S2W2, HG1S3W3 and HG2S2W1. The per cent of reduction in chlorophyll 'b' was 71.8 to 74.8 compared to mean value. The treatment combination of HG2S1W1 was statistically non- significant. The frequency distribution of treatment combination indicates that 40.1 per cent of treatment combinations had value less than 0.4239 and 33 per cent had more than 0.5091 mg g^{-1} fresh leaf (Table 12b).

Significant variation existed among the treatment combinations of total chlorophyll content for all levels. Table 11 shows the data on total chlorophyll for all levels. The mean total chlorophyll content observed under open condition was 1.3913 mg g^{-1} fresh leaf and the range in total chlorophyll was from 0.51 to 2.22 mg g^{-1} fresh leaf. The significant reduction of total chlorophyll content

Table 12a: Grouping of 27 treatments and their frequency distribution for Chlorophyll 'a' content under different shade condition.

Chlorophyll 'a' under open				Chlorophyll 'a' under fifty per cent shade			Chlorophyll 'a' under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	>0.93	1,4,5,7,8,9,14,15,18,20,21,22,23,	-13-	>1.25	9	-1-	>1.13	21,23,27,26,	-8-
Mean ± SE	0.93-1.11	Rest of the treatment	-5-	1.25-1.48	Rest of the treatment	-16-	1.13-1.34		
Mean+SE	<1.11	10,11,12,13,16,17,19,24,26	-9-	<1.48	2,4,5,6,8,9,10,11,12,14,	-10-	<1.34	4,5,6,7,8,9,10,11,12,14,17,19,22,25	-14-

Table 12b: Grouping of 27 treatments and their frequency distribution for Chlorophyll 'b' content under different shade condition.

Chlorophyll 'b' under open				Chlorophyll 'b' under fifty per cent shade			Chlorophyll 'b' under seventy five per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<0.33	1,2,3,4,5,6,7,8,18,20,21, 22,23, 27,	-12-	<0.58	4,10,12,13,14,15, 16,17,18,19,20,21,22,24,26,27	-15-	<0.42	1,3,4,5,11,12, 13,17,18,23, 26,	-11-
Mean ± SE	0.33-0.40	Rest of the treatments	-5-	0.58-0.69	Rest of the treatments	-2-	0.42-0.50	Rest of the treatments	-8-
Mean+SE	>0.40	6,1011,12,13,15,16, 23,25,	-10-	>0.69	6,10,11,12,13,15, 16,19,23, 25,	-10-	>0.50	2,6,7,8,10,14, 21,	-8-

Table 12 c: Grouping of 27 treatments and their frequency distribution for Chlorophyll 'Total' content under different shade condition.

	Chlorophyll 'Total' under open			Chlorophyll 'Total' under fifty per cent shade			Chlorophyll 'Total' under seventy per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<1.33	1,4,5,7,8, 18,20,21,22,2 3,27	-11-	<1.92	10,11,112,14, 15,17,18,19,2 0,21,22,25,26, 27	-15-	<1.63	1,3,5,9,13,15, 20,21,23,25, 27	-11-
Mean ± SE	1.33- 1.45	Rest of treatments	-7-	1.92- 2.08	Rest of treatments	-2-	1.63- 1.78	Rest of treatments	-1-
Mean+SE	>1.45	3,10,11,12,13, 16,19,24,26	-9-	2.08	1,3,2,4,6,7,8,9 ,23,24,	-10-	>1.78	2,4,6,7,8,10,1 1,12,14,15,16, 17,18,22,24	-15-

among treatment combination of OG1S2W1 and OG3S3W3 were observed .The per cent of reduction was 63 compared to the mean value. The treatment combinations of OG3S2W3 and OG3S3W2 were non significant. The frequency distribution of treatment combination indicates that 41 per cent of treatment combinations had value less than 1.33 mg g^{-1} fresh leaf and 33 per cent of treatment combination had more than 1.45 mg g^{-1} fresh leaf respectively (Table 12c).

In fifty per cent of shade, the overall mean and range were 2.0054 and 1.22 to 2.82 mg g^{-1} fresh leaf respectively. There was significantly reduction on content of treatment combinations of total chlorophyll content were FG3S1W1 and FG3S2W1.The per cent of reduction was 39 per cent compared to mean value. The treatment combination of FG1S3W1 and FG2S3W3 were non significant. The frequency distribution of treatment combinations indicates that 55 per cent of treatment combinations had less than 0.9217 mg g^{-1} fresh leaf and 37 per cent of had more than 2.089 mg g^{-1} fresh leaf (Table 12c).

In seventy five per cent of shade level, the mean total chlorophyll was 1.709 mg g^{-1} fresh leaf and the range in total chlorophyll was from 0.66 to 2.66 mg g^{-1} fresh leaf. The treatment combination in which significantly reduced of total chlorophyll content was HG3S3W3. The per cent of reduction was 46 compared to mean value. The frequency distribution of treatment combinations reveals that 40 per cent of treatment combinations had value less than 1.636 mg g^{-1} fresh leaf and 35 per cent of had more than 2.0891 mg g^{-1} fresh leaf (Table 12c).

4.2.4 Membrane Stability index

Membrane integrity varied significantly among 27 treatment combinations under all shade and open conditions (Table.13). In open condition, the overall mean and range were 51.81 and 40.4 to 80.38 per cent respectively. The Significant reduction in membrane stability index was observed in the treatment combinations of OG1S1W3. The reduction of membrane stability was 92 per cent compared to mean value. The non significant treatment combinations were

Table 13. Effect of abiotic stress on Membrane stability index of *Andrographis paniculata* under different shade condition

Sl. No.	Treatment	Membrane stability Index (%)		
		Open	50% shade	75% shade
1	G1S1W1	36.90	44.19	18.43
2	G1S1W2	34.15	67.60	24.76
3	G1S1W3	33.64	55.14	26.71
4	G1S2W1	67.36	70.24	21.42
5	G1S2W2	29.50	63.35	17.13
6	G1S2W3	72.89	90.81	27.21
7	G1S3W1	58.35	33.50	25.19
8	G1S3W2	34.72	37.57	17.16
9	G1S3W3	40.82	59.57	28.97
10	G2S1W1	71.57	54.23	26.97
11	G2S1W2	60.91	93.93	28.06
12	G2S1W3	21.04	63.24	36.35
13	G2S2W1	58.25	56.05	22.60
14	G2S2W2	79.50	41.37	29.55
15	G2S2W3	61.38	47.86	11.83
16	G2S3W1	61.62	56.24	34.94
17	G2S3W2	54.81	69.87	14.43
18	G3S3W3	24.04	87.08	25.74
19	G3S1W1	79.49	52.72	35.26
20	G3S1W2	80.38	20.31	29.13
21	G3S1W3	35.98	38.19	15.68
22	G3S2W1	71.36	27.47	75.64
23	G3S2W2	24.58	21.82	13.06
24	G3S2W3	30.78	50.14	49.18
25	G3S3W1	61.85	45.97	17.63
26	G3S3W2	48.62	46.12	19.18
27	G3S3W3	64.47	44.14	24.68
	Mean	51.81	53.28	26.55
	CD (5%)	4.49	4.72	2.40

Table 13a: Grouping of 27 treatments and their frequency distribution for Membrane stability index under different shade condition.

Membrane stability index under open				Membrane stability index under fifty per cent shade			Membrane stability index under seventy per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<48.94	1,2,3,5,8,9,11,18,21,23,24,26	-12-	<51.66	1,7,8,14,15,20,21,22,23,24,25,26,27	-13-	<24.55	4,5,8,15,17,21,23,25,26,27	-10-
Mean ± SE	48.94-53.4216	Rest of treatments	-1-	51.66-56.382	Rest of treatments	-3-	24.55-27.74	Rest of treatments	-9-
Mean+SE	>53.4216	4,6,7,10, 11,13,14, 15,16,17, 19,20,22,25	-14-	>56.382	2,4,5,6,9,11, 12,13,16,17, 18,	-11-	>27.74	2,9,11,12,14, 13,19,22,24	-8-

OG2S2W2, OG2S2W1 and OG3S1W2.The frequency distribution of treatment combinations indicates that 44 per cent of treatment combination had value less than 51.8 per cent of membrane stability index and 52 per cent had more than 53 per cent of membrane integrity (Table 13a).

Under fifty per cent of shade level, mean value of 54.02 was observed and the range of membrane integrity was 20.31 to 110.81 per cent. There was a significant reduction in treatment combination of FG3S1W2 and FG3S2W2. The reduction of membrane stability index was 62 per cent compared to mean value. The non significant treatment combination was FG1S2W3. The frequency distribution of treatment combination indicates that 48 per cent of treatment combination had membrane stability index of less than 52 per cent and 40 per cent had more than 56 per cent (Table 13a).

In seventy five per cent of shade level, the membrane integrity of plants varied from 11.83 to49.18 per cent with mean value of 26.55 per cent. The treatment combinations of HG2S2W3 and HG3S2W2 membrane stability index were reduced significantly. The per cent reduction in membrane stability index was 50 to 55 per cent compared to mean value. The non significant treatment combination was HG3S2W1. The frequency distribution of treatment combination indicates that 31 per cent of treatment combination had membrane stability index less than 24 per cent and 30 per cent had more than 27.7 per cent (Table 13a).

4.3 BIOCHEMICIAL CHARACTERS

4.3.1 Andrographolide Content (per cent)

Andrographolide content showed significant variation among stress treatment combinations under open and different shade levels (Table.13). The treatment combinations of OG1S3W2 and OG3S3W2 showed significantly increased levels of andrographolide contents. The mean andrographolide content was 0.75 per cent and the range in andrographolide content under open level was from 0.24 to 1.35 per cent .The per cent of increase was 70 to 79 per cent compared to mean value. The treatment combination of OG2S2W3 was statically

Table 14. Effect of abiotic stress on andrographolide content of *Andrographis paniculata* under different condition (per cent).

Sl. No.	Treatment	Andrographolide content		
		Open	50% shade	75% shade
1	G1S1W1	0.55	0.56	0.29
2	G1S1 W2	0.57	0.59	0.35
3	G1S1W3	0.62	0.34	0.22
4	G1S2W1	1.05	0.58	0.07
5	G1S2W2	0.42	0.57	0.18
6	G1S2W3	0.33	0.32	0.29
7	G1S3W1	1.00	0.26	0.35
8	G1S3W2	1.28	0.31	0.19
9	G1S3W3	0.94	0.22	0.09
10	G2S1W1	0.47	0.18	0.09
11	G2S1W2	0.44	0.22	0.08
12	G2S1W3	0.56	0.30	0.14
13	G2S2W1	0.38	0.42	0.09
14	G2S2W2	0.77	0.26	0.15
15	G2S2W3	0.24	0.45	0.11
16	G2S3W1	0.61	0.47	0.10
17	G2S3W2	0.92	0.86	0.14
18	G2S3W3	1.00	0.87	0.16
19	G3S1W1	1.15	0.20	0.15
20	G3S1W2	0.43	0.17	0.14
21	G3S1W3	0.50	0.18	0.07
22	G3S2W1	0.92	0.20	0.02
23	G3S2W2	0.95	0.14	0.00
24	G3S2W3	1.07	0.12	0.04
25	G3S3W1	1.10	0.27	0.02
26	G3S3W2	1.35	0.31	0.00
27	G3S3W3	0.70	0.35	0.08
	Mean	**0.75**	**0.35**	**0.13**
	CD (5%)	**0.06**	**0.03**	**0.013**

Table 14a: Grouping of 27 treatments and their frequency distribution for Andrographolide content under different shade condition.

Andrographolide content under open				Andrographolide content under fifty per cent shade			Andrographolide content under seventy per cent shade		
Status	Range	Entries	Frequency	Range	Entries	Frequency	Range	Entries	Frequency
<Mean- SE	<0.71	1,2,3,5,6, 10,11,12, 13,15,16, 20,21,27	-14-	<0.34	5,6,7,8,9,10, 11,14,19,20, 21,22,23,24, 25,	-15-	<0.12	4,9,10,11,15, 16,21,22,23, 24,25,26,27, 28,	-14-
Mean ± SE	0.71-0.78	Rest of treatments	-1-	0.34-0.37	Rest of treatments	-3-	0.12-0.14	Rest of treatments	-4-
Mean+SE	>0.78	7,8,9,17, 18,19,20, 22,23,24, 25,26	-12-	>0.37	1,2,4,5,13,15,16 ,17,18,	-9-	>0.14	1,2,3,5,6,7,8, 18,19	-9-

non- significant. The frequency distribution of treatment combinations indicates that 32 per cent of treatment combination had andrographolide content of less than 0.71 per cent and 44.4 per cent had more than 0.785 per cent (Table 14a).

In fifty per cent of shade level, the overall mean and range were 0.35 per cent and 0.12 to 0.57 per cent respectively. The significantly increase in andrographolide content was observed under treatment combinations of FG2S2W1 and FG2S3W1 where the per cent of increase was 142 per cent compare to mean value .The treatment combinations of HG2S3W2 and HG3S3W3 were statistically non-significant. The frequency distribution of treatment combination indicates that 55 per cent of treatment combinations had andrographolide content of less than 0.34 per cent and 33 per cent treatment combination had more than 0.37 per cent (Table 14a).

In seventy five per cent shade level, the mean andrographolide content of 0.1336 per cent was observed and range in andrographolide content was from 0.01 to 0.35 per cent. The significant treatment combinations were HG1S1W2 and HG1S3W1 where the per cent of increase was 161 compare to mean value. The non significant treatment combinations were HG3S2W2 and HG3S3W2. The frequency distribution of treatment combination indicates that 52 per cent of treatment combination had andrographolide content of less than 0.12 per cent and 33 per cent had more than 0.14 per cent respectively (Table 14a).

4.3.2 Pooled Analyses of Biochemical Characters

Under each shade level, five treatments were selected for doing the biochemical analysis. Selection was based on the andrographolide content. Among the five treatments, four were having higher andrographolide content and one was of the lowest andrographolide content under each shade level.

4.3.2.1 Starch Content

The data on the effect of abiotic stress on starch content of *Andrographis paniculata* is presented in Table 15. The treatment combination of HG1S3W2 (6.47 mg /g) recorded the maximum value and the treatment combinations of

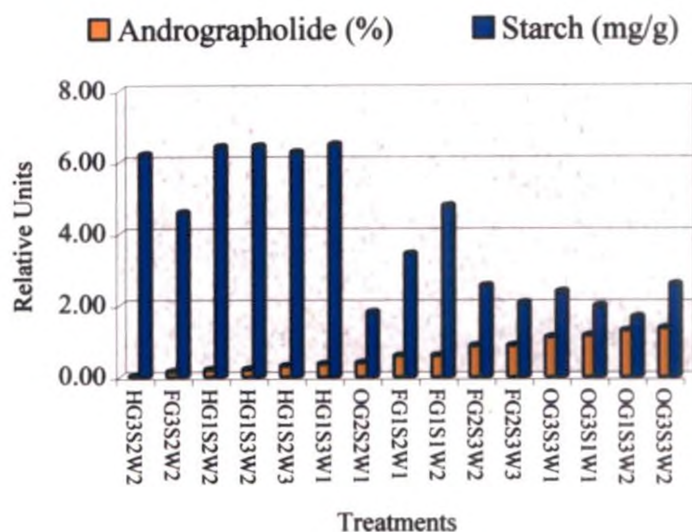


Fig. 10. Relationship between the androgrpholide content and starch content

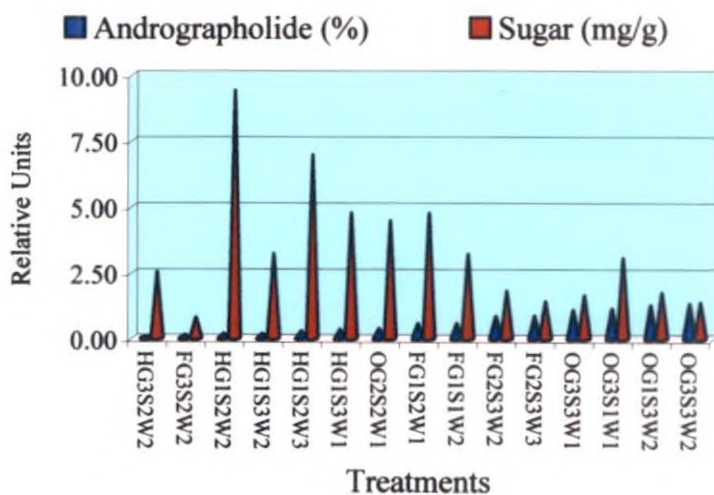


Fig. 11. Relationship between the andrographolide content and sugar content

Table 15. Effect of abiotic stress on carbohydrates fractions in *Andrographis paniculata*

Sl. No.	Treatment	Starch	Sugar
1	OG1S3W2	1.66	1.76
2	OG3S3W2	2.56	1.40
3	OG3S1W1	1.96	3.08
4	OG3S3W1	2.36	1.66
5	OG2S2W1	1.79	4.48
6	FG2S3W3	2.05	1.43
7	FG2S3W2	2.53	1.83
8	FG1S2W1	3.42	4.77
9	FG1S1W2	4.76	3.21
10	FG3S2W2	4.57	0.81
11	HG1S2W2	6.41	9.40
12	HG1S2W3	6.25	6.96
13	HG1S3W1	6.47	4.76
14	HG1S3W2	6.43	3.23
15	HG3S2W2	6.20	2.54
	Mean	**3.96**	**3.42**
	CD (5%)	**0.36**	**0.34**

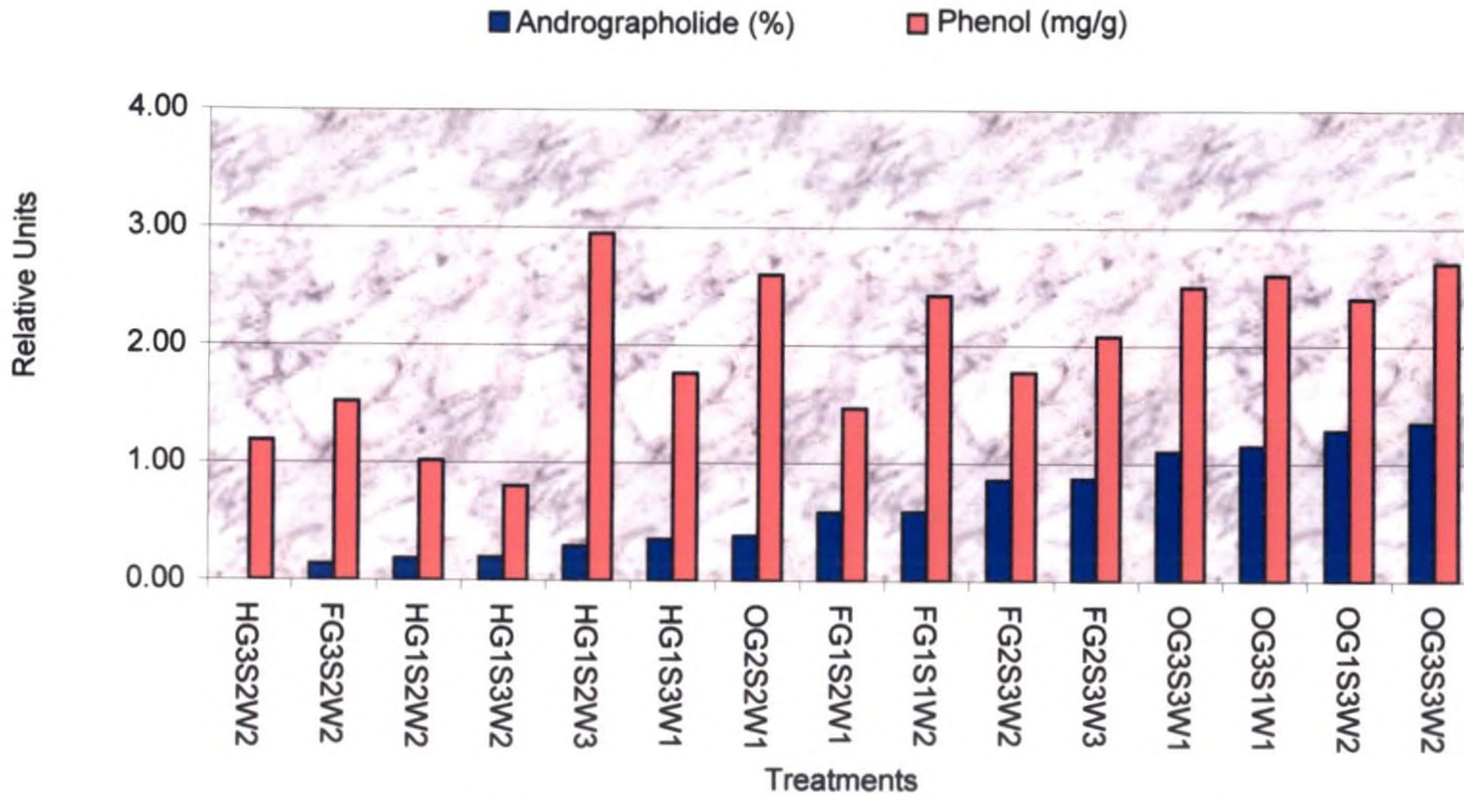


Fig. 12. Relationship between the andrographolide content and phenol content

OG1S3W2 (1.66) mg /g was recorded the minimum. The significant treatments were HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2 and HG1S2W2. However, the starch content in other treatment combinations was on par with the mean value. The starch and andrographolide content showed an inverse relationship between them. In the open condition, the starch content was less and there was higher content of andrographolide content. Under fifty per cent shade, the starch content was increased compared to that under open condition corresponding decrease in the andrographolide content was observed (Fig.10). Maximum value for starch content and minimum value of andrographolide content were recorded under 75% shade.

4.3.2.2 Reducing Sugar Content

The data on sugar content as influenced by abiotic stress treatment is given in Table 15. Analysis of data revealed statistical significance among the treatments. The treatment combination of FG3S2W2 (0.81 mg/g) recorded the minimum value and HG1S2W3 (9.40 mg/g) recorded the maximum value. Other treatment combinations were on par with mean value. There was an inverse trend between the sugar and accumulation of andrographolide contents. In the open condition, the sugar content was decreased and a corresponding increase was seen in andrographolide content (Fig.11). The same trend was observed under the fifty per cent and seventy five per cent shade levels also.

4.3.2.3 Phenol Content

The data on the effect of abiotic stress on phenol content of *Andrographis paniculata* is presented in Table 16. Analysis of data revealed statistical significance among the treatment combinations. The significant treatment combination was HG1S2W3 recorded 2.9 mg/g. The minimum value was for HG1S3W2 (0.8 mg/g). Other treatment combinations were found to be on par with mean value. The relationship between phenol and andrographolide content was almost direct or positive them (Fig.12). There were two peaks in the phenol content at fifty and seventy five per cent of shade where corresponding increase in andrographolide content was also increasing.

Table16. Effect of abiotic stress on Protein fractions and phenols in *Andrographis Paniculata*

Sl. No.	Treatment	Protein	Amino acids	Phenol	Ascorbic acid
1	OG1S3W2	1.14	1.92	2.4	2.1
2	OG3S3W2	2.47	0.69	2.7	1.3
3	OG3S1W1	2.66	1.08	2.6	1.4
4	OG3S3W1	3.24	0.69	2.5	1.8
5	OG2S2W1	3.57	1.25	2.6	1.6
6	FG2S3W3	3.68	0.24	2.1	1.4
7	FG2S3W2	3.26	0.98	1.8	1.2
8	FG1S2W1	2.54	0.23	1.5	1.0
9	FG1S1W2	7.48	0.82	2.4	1.5
10	FG3S2W2	3.37	0.18	1.5	1.6
11	HG1S2W2	5.90	0.23	1.0	1.2
12	HG1S2W3	7.67	0.80	2.9	0.8
13	HG1S3W1	4.42	1.33	1.8	0.9
14	HG1S3W2	6.71	1.59	0.8	0.7
15	HG3S2W2	3.37	0.18	1.2	0.9
Mean		**4.09**	**0.81**	**1.98**	**0.6**
CD (5%)		**0.37**	**0.08**	**0.15**	**1.33**

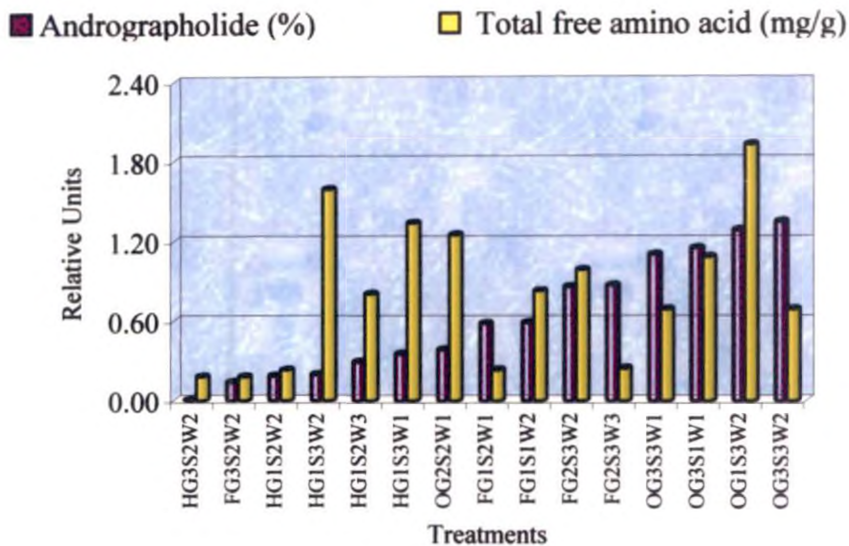


Fig.13. Relationship between andrographolide content and total free aminoacid

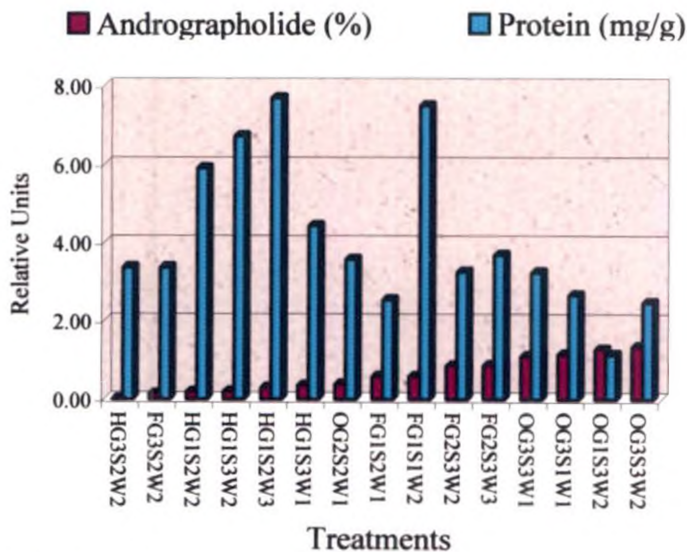


Fig. 14. Relationship between the andrographolide content and protein content

4.3.2.4 Total Free Amino Acids Content

The effect of abiotic stress on the amino acid content was shown in Table 16. Among the treatment combination, OG1S3W2 (1.92 mg/g) had the maximum .The statistically non-significant treatment combinations were FG2S3W2, FG1S2W1, FG3S2W2, HG1S2W2 and HG3S2W2. There was no relationship between andrographolide content and total amino acid was not showing any relation (Fig.13).

4.3.2.5 Total Protein Content

The effect of abiotic stress on the protein content is represented in Table 16. Among the treatments, FG1S1W2 (7.48 mg/g) had the maximum value and the minimum value was recorded by OG1S3W2 (1.14). The statistically non-significantly treatment combination was OG1S3W2. The results revealed that plants grown under shade levels had more protein content than open condition. There was negative relationship between the andrographolide content and the protein content (Fig.14). There was a decrease in the andrographolide content and a corresponding increase protein under both fifty and seventy five per cent of shade levels

4.3.2.6 Ascorbic acid

The data on the ascorbic acid in plants subjected to various stress combinations were given in Table 16. The ascorbic acid was high in open condition. It was low under shade condition. The statistically significant treatments were HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2 and HG3S2W2. The non significant treatments were OG1S3W2.

4.3.2.7 Catalase Activity

The data on catalase activity in plants subjected to various combinations of stress is given in Table 17. Analyses of data revealed that the treatments statistically significant variation existing among the various treatment combinations. FG2S3W3 had maximum value (607.14 $Eul^{-1}hr^{-1}$) and treatment combination of OG3S2W2, OG3S3W2, OG2S2W1, FG3S2W2 were non

Table17. Effect of abiotic stress on Antioxidant enzymes in *Andrographis paniculata*

Sl. No.	Treatment	Catalase	Peroxidase	SOD
1	OG1S3W2	369.57	238.10	1.56
2	OG3S3W2	377.78	657.89	6.35
3	OG3S1W1	333.33	285.71	5.66
4	OG3S3W1	354.17	434.78	6.21
5	OG2S2W1	333.33	645.16	2.74
6	FG2S3W3	607.14	135.50	1.86
7	FG2S3W2	548.39	124.69	1.79
8	FG1S2W1	377.78	68.87	1.98
9	FG1S1W2	386.36	66.53	1.98
10	FG3S2W2	326.92	54.00	5.98
11	HG1S2W2	472.22	75.59	1.47
12	HG1S2W3	531.25	76.80	1.54
13	HG1S3W1	447.37	76.75	1.48
14	HG1S3W2	500.00	80.65	1.56
15	HG3S2W2	404.76	70.18	1.79
	Mean	**424.69**	**206.08**	**2.93**
	CD (5%)	**36.11**	**24.08**	**0.58**

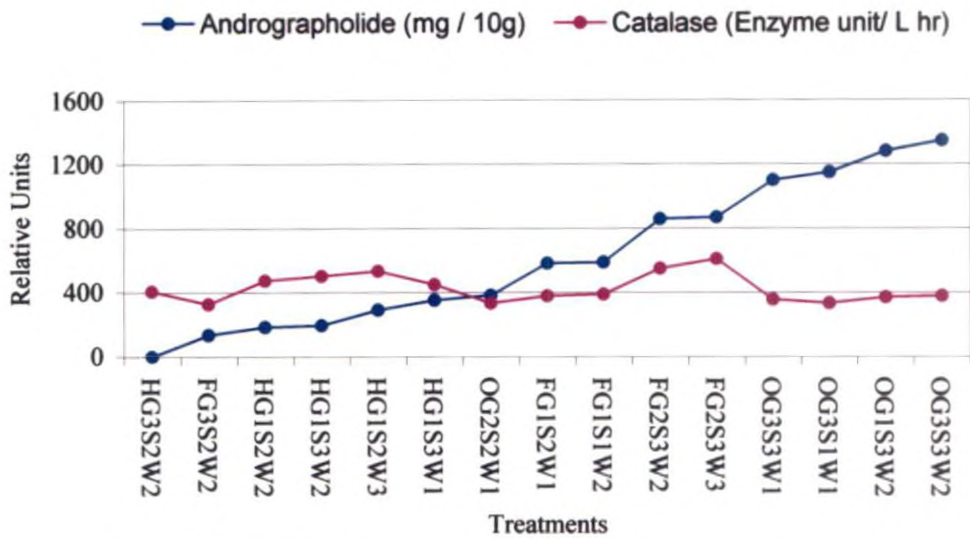


Fig. 15. Relationship between the andrographolide content and catalase activity

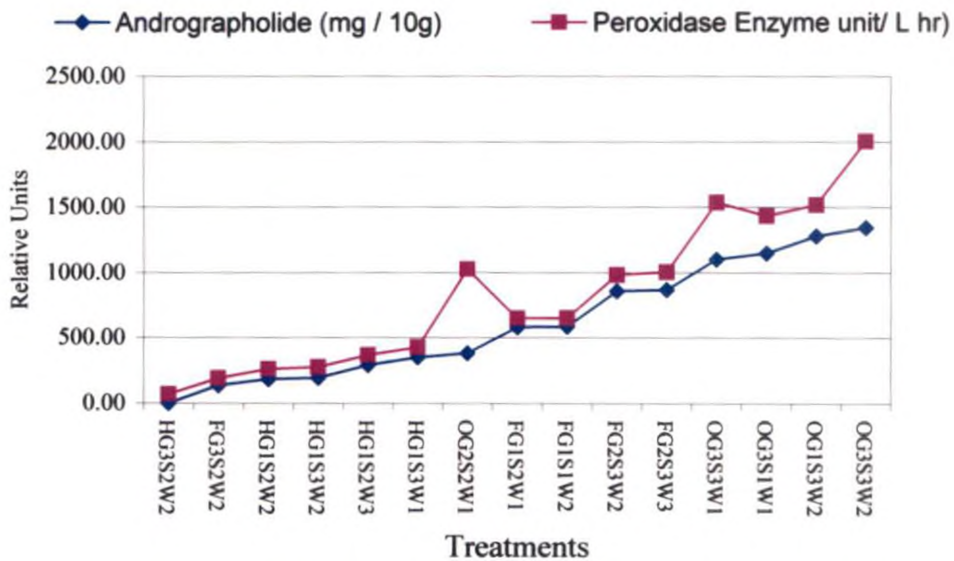


Fig. 16. Relationship between the andrographolide content and peroxidase activity

significant. The relationship between the catalase activity and andrographolide content was to be a direct (Fig.15).

4.3.2.8 Peroxidase Activity

The data on peroxidase activity of plants subjected to various combinations of stress is given in Table 17. Analysis of data reveals that statistically significant. OG3S3W2 and OG2S2W1 had maximum values of peroxidase activity and treatment combinations of FG2S3W3, FG2S3W2, FG1S2W1, FG3S2W2, HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2, HG3S2W2, were non significant. The relationship between peroxidase and andrographolide content was positive. In the shade condition, both peroxidase and andrographolide content was increased (Fig.16).

4.3.2.9 Superoxide Dismutase

The data on superoxide dismutase (SOD) activity of plants subjected to various stress is given in Table 17. In the present study, treatment combinations of OG3S3W2, OG3S3W1 and FG3S2W2 were statistically significant. The SOD activity was increased under salinity and water stress combinations. The non significant treatment combinations were OG1S3W2, FG2S3W3, FG2S3W3, FG2S3W2, FG1S2W1, FG1S1W2, HG1S2W2, HG1S2W3, HG1S3W1, HG1S3W2 and HG3S2W2. Compared to shade condition, the SOD activity was high in open condition and was very low under seventy five per cent condition. There was no relationship between the andrographolide content and SOD activity (Fig.17).

4.4 GENE EXPRESSION

4.4.1 Effect of Stress on the Expression of HMGR.

Three treatments have been selected for studying the differential expression of HMGR, the key enzyme for mevalonate pathway of IPP biosynthesis. There was distinct variation in the expression levels of HMGR between treatments as evidenced from the RT-PCR (Fig 19). Plants grown under 50% shade without any stress had higher expression and for plants grown at 90

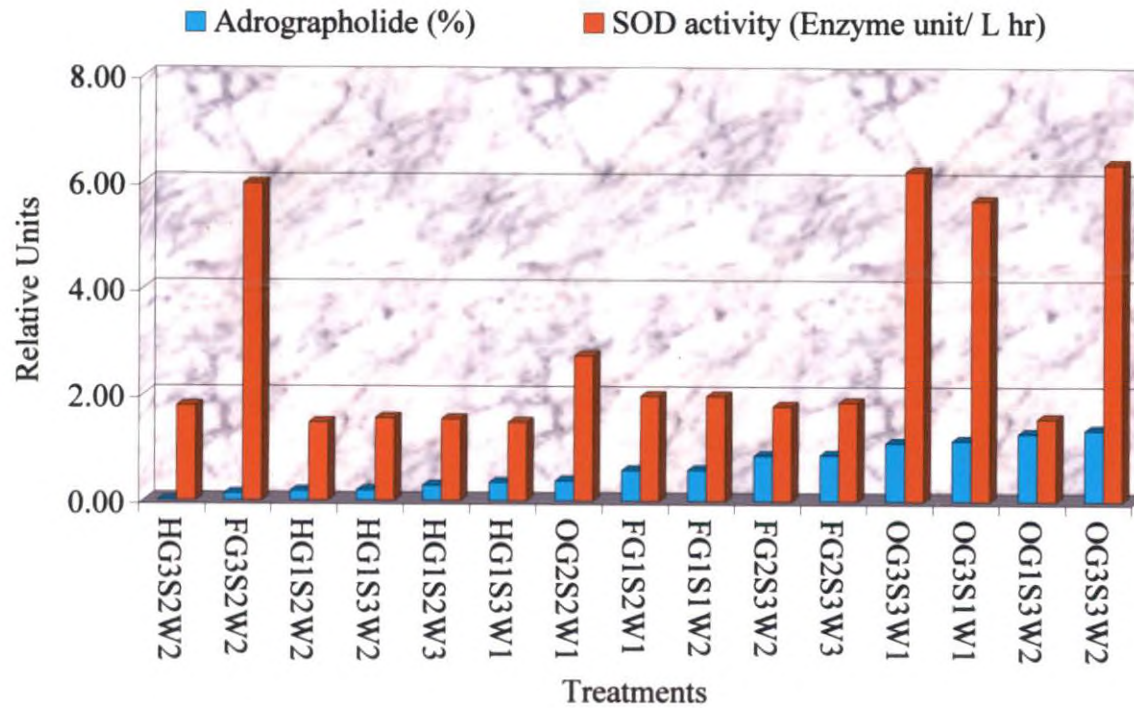


Fig.17. Relationship between the andrographolide content and Superoxide dismutase (SOD)

DAS with 6 mmhos/cm salinity and 30% FC had lower accumulation of HMGR mRNA . The plants grown under open with a combination of water stress (60%FC) and salinity (6mmhos/cm) also had lower expression level in the quantitative RT-PCR, expression analysis.

Discussion

5. DISCUSSION

The overall pattern of plant growth and development are directed by two classes of regulatory signals *viz.*, ontogenic signals, programmed by the genome and environmental signals that provide external cues for continued action of the ontogenic signals. Response to developmental and environmental cues occur by stimulus -response coupling.

The plant secondary metabolites have a pivotal role in the ecophysiology of plants, mainly as protectants to biotic and abiotic stresses. Hence variations in the environmental factors alter the secondary metabolism and change the levels of medicinally active compounds.

An increase in the synthesis of secondary compounds was a common response to environmental stress in plants (Dixon and Paria, 1995). Therefore attempts to identify stress factors causing differences in secondary metabolite production and identifying morphological and physiological traits associated with higher secondary metabolite production gained considerable importance in recent years. The information regarding of accumulation or depletion of different phytomedicinal compounds during environmental stress will be useful to facilitate change in specified environmental area.

With this view in mind, present investigation was undertaken to study the effect of critical stress treatment combinations to produce higher andrographolide content and understand the various physiological and biochemical changes causing the increased production of andrographolide and to analyse the differential expression of HMGR reductance the key gene of regulating the isoprenoids biosynthesis in plant by different abiotic stress and developmental stages. The result of various experiments conducted to address the above objectives in this chapter with sufficient support from earlier studies.

5.1 EFFECT OF ABIOTIC STRESS ON BIOMETRIC CHARACTERS OF *ANDROGRAPHIS PANICULATA*

The effect of abiotic stresses *viz.*, 3 levels of salinity (control, 3mmhos/cms and 6mmhos/cm) and there levels of water stress (100%FC, 60%FC and 30%FC) which were given for a period of 30days to the plants. The plants were at three different biometric shade levels *viz.*, open, 50% shade and 75% shade. Observations on three different biometric characters were analyzed. From that data on the biomass and leaf area and other growth characters like NAR, RGR etc are also calculated. The effects of stress like shade, water stress and salinity at different development of stag of plants on the biometric characters are discussed in the following section. The effect of stress like shade levels on plant height was significant. An increasing trend in plant height with increasing intensity of shade from open to 50% was observed followed by a reduction in height at 75%. The plants grown under open condition registered lower plant height in all growth stages. This finding was in agreement with the results reported by Ancy (1992) and Babu (1992).

Jensen *et al.* (1998) reported that auxin transport plays an important role in stem elongation. Behringer and Davis (1992) concluded that phytochrome regulation of stem elongation is partly the result of changes in IAA levels. Martinez-Gracia and Gracia-Martinez (1992) found that stem elongation might be strongly influenced by Red/FarRed involving the phytochrome system, which is mediated through increased levels of gibberellins leading to cell elongation. Shading resulted in a change of gibberellin concentration in plants, which in turn resulted in the higher plant height under shade condition. Increase in plant height is the result of phytochrome mediated shade avoidance of the plants (Lambers *et al.*, 1998).

The water stress at 60% FC and 30% FC caused a reduction in plant height in all shade levels and growth stages. Gregorg *et al.* (1987) reported that reduction of plant height under drought was due to reduced stem growth, cell

elongation and reduced photosynthesis. The primary plant plant process affected by water stress is the cell elongation as a result of reduced turgor pressure and reduction in cell elongation as a result of reduced turgor pressure and reduction in cell expansion in the basic cause for reduced plant height under water stress (Taiz and Zeiger, 2003).

Salinity also reduced the plant height in all shade levels. The reduction of plant height was due to altered water relations in cells and spending of more ATP for osmotic adjustment, which could be used for growth (Passioura and Munns, 2000). At cellular level, the consequences of NaCl building in the cell wall are catastrophic. As the concentration is increases the cell will shrink and thus elongation of cells (Munns, 2002). This reduction in cell elongation is cause of reduced plant under salinity under all shade levels. When plants were to expose to a combination of water stress and salinity, the reduction in plants was drastic since both stress would cause a reduction in the cell expansion or cell elongation.

The drymatter production at all growth stages was significantly influenced by the shade. In general, maximum dry matter was recorded in plants under 50% shade followed by plants grown under open. However, the lowest dry matter production was observed in 75% shade. The result of the present study was in accordance with the findings of Ancy (1992) and Babu (1992), who also reported an increase in dry matter production under shade condition in ginger.

Dry matter production was highest under 50% shade because the plants had higher assimilatory surface area which enhanced the total photosynthetic output per plant under this condition. The reason for low dry matter under 75% shade was the prevalence of very low light intensity which turned into limiting light stress and further reduced the photosynthesis. So it is inferred from the present findings based on the performance of plants under different light that *Andrographis panicualata* is adapted to partial shade condition and have a low light saturation point. Lambers *et al.*,(1998) have reported that the phytochrome A is responsible for the acclimation of the CO_2 assimilation under the shade

condition and also the light harvesting aspects of the photosynthesis with a change in the chlorophyll a and b content. The plants under open had lower dry matter production because of the excess irradiance and some of the photons observed by the chlorophyll were not used in the phytochemistry resulting in the temporarily reduced quantum yield of the photosynthesis. The excess excitation might cause damage to the photosynthetic membrane causing the photoinhibition and photooxidation due to the generation of reactive oxygen species.

Dry matter production was decreased with increasing water content from 30 % FC to 100% FC. This reduced dry matter under water stress might be due to decrease in number of branches and reduced leaf area. The photosynthesis might be reduced initially because of the higher stomatal resistance caused due to water stress. The relative part of the stomatal limitation of photosynthesis is depending on the severity of water stress. When plants were exposed to water stress the first line of defense is the stomatal closure because of chemical signal ABA produced by roots in contact with the dry soil is transported to the leaves, thus reducing the tranpiratory water loss. This causes a proportionate decrease in the assimilation rate and thus the response of water to plants will be reduction in leaf area and total biomass accumulated.

The dry matter production was also reduced in high salinity treatments under all shade levels. The salinity might have caused damage to photosynthetic machinery and resulted in reduced photosynthetic rate. This in turn leads to probably low dry matter production under saline condition. The stomatal resistance to Co_2 entry was also high under salinity because of lower water availability owing to salt induced reduction in soil water potential. Bandeoglu *et al.* (2004) have reported that the most typical symptom of saline injury to a plant is retarded growht due to inhibition of cell elongation. They also observed around 50% reduction in growth parameters in response NaCl stress (EC of 3 dsm^{-1}).

Andrographis paniculata grown under 50 and 75 per cent shade levels

produced higher leaf area. In general, plants grown under 50% shade recorded maximum leaf area. The lowest leaf area was registered when plants were grown under open condition. The result was in accordance with the study of Pratima, (1998). The sun leaves are thicker due to the formation taller palisade cells and increased in the number of the layer of palisade cells and hence we have lesser area since light is not limiting. The increased leaf area under shade was due to the plant adaptation to expose larger photosynthetic surface under limited illumination.

Leaf area was reduced significantly under 60% FC and 30% FC conditions water stress inhibition of cell expansion resulted in reduced of leaf expansion, for effectively conserving water by reducing transpiration because of a limited water supply in the soil over a period of time (Taiz and Zeiger, 2003). One of the mechanism of water stress tolerance is to reduce transpirational surface area which hwlps the plants to reduce the heat load on the leaves and reduce the generation ROS.

Salinity depresses the leaf area in all stages and shade levels. Decreasing trend of leaf area was observed with increasing levels of salinity. This is due to reduced photosynthetic rate and growth (Passioura and Munns, 2000). Salinity also reduced cell expansion resulting in reduced leaf area. Bandeoghu *et al.* (2004) have also found leaf area reduction in response to salinity and reported that it was due to salinity-induced inhibition of cell elongation.

An incresing trend in leaf area duration was noted from open to 50% shade but under 75% it was declined. The decreased LAD could reduce photosynthesis under shade (Quirino *et al.*, 2000) and in turn resulted in low dry matter accumulation. The accelerated senescence because of very light intensity at 75% shade and also contribute lower LAD.

Leaf area duration decreased with water stress from 100% FC to 30% FC in all growth and shade levels. This was due to restricted leaf area expansion, accelerated senescence and abscission of leaves. These mechanisms will reduce

the heat load on plants by reducing the leaf area and help the plants and survive in water stress condition (Lamberts *et al.*, 1998)

The leaf area duration was reduced under salinity and also in all shade levels. Decreasing trend of leaf area was observed with increasing the salinity from 3mmhos/cm to 6mmhos/cm. The reduction of LAD was due to the spending of more ATPS for osmotic adjustment which would otherwise be used for growth. Salinity induced accelerated leaf fall and reduced leaf expansion also led to reduction in LAD (Taiz and Zeiger, 2003).

The specific leaf area was high in 50% shade and was very low in open condition. The lower value of the specific leaf area in open condition was due to lesser leaf area and higher leaf weight. The shade plants have thin and a broad leaf to harvest maximum light since light is the limiting factor under shade. Such leaves will have higher thylakoids per stroma volume of many thylakoids per granum. The palisade parenchyma thickness will be low in shade leaves where as under open condition the leaf thickness is more and they will have more cell layers in the leaf to have more enzymes for utilizing the assimilatory power generated due to high light intensity (Lambers *et al.*, 1998)

The effect of shade levels on RGR and NAR was found to be significant. An increasing trend in RGR and NAR with increasing shade intensity from open to 50% shade was observed. But there was decrease in RGR and NAR under higher shade (75%) condition. Under shade due to unavailability of sufficient light intensity the crops were not able to synthesis assimilates efficiently leading to lesser NAR and RGR (Taiz and Zeiger, 2003). Under open condition, the photosynthetic capacity per unit area of leaf will be higher compared to that in extreme shaded plants. Under such extreme shade condition, there will be lesser Rubisco content, lesser nitrogen and enzymes and also less chlorophyll per chloroplast compared to open condition (Lambers *et al.*, 1998).

Water stress significantly reduced RGR and NAR in all shade levels and growth stages. The reduction in RGR and NAR is attributed to the reduction in

cell elongation, expansion and volume, which resulted due to lower turgor pressure. Since turgor reduction is the earliest significant biophysical effect of water stress, turgor-dependent activities such as leaf expansion and root elongation were inhibited under stress condition (Taiz and Zeiger, 2003)

The effect of salinity levels on RGR and NAR was found to be significant. The reduction in NAR and RGR was due to change in water relations in cells as well as spending of more ATP for osmotic adjustment, which should have been used for growth. Salinity induced reduction in leaf area also contributed to the reduction in growth rates. Bandeoglu *et al.* (2004) reported that under higher saline condition the relative growth rate was decreased in rice.

The chlorophyll a, b, and total contents increased under shade condition. There was increasing trend noted with increase in the shade intensity. According to Lambers *et al.,* (1998) under shade, chloroplasts in general produced larger grana, which contain major part of the chlorophyll. This might have been the reason for higher chlorophyll content under shaded condition, which is an adaptation of these plants to harvest more radiation. However, due to reduced calvin cycle enzymes there is a chance of reduced photosynthetic accumulation which might be the reason for the observed reduction in the dry matter production.

Water stress significantly reduced the chlorophyll content. The reduction of the chlorophyll was mainly due to cellular disruption of thylakoids and the chloroplast swelling breaking down under drought condition (Walter, 1995). The water stressed induced generation of ROS also damaged the membrane and causes the photoxidation. Thus reducing the reducing the chlorophyll contents under water stressed (Lambers *et al.,* 1998)

Under salinity, there was no distinct trend noted regarding chlorophyll content with the intensity of stress. But there was significant reduction in chlorophyll content. This would have resulted due to breakdown of chloroplast or production of anthocyanin under saline condition (Walter, 1995).

The Membrane stability index was almost similar in open and 50% shade condition. But it was decreased in 75% shade. In open and 50% shade condition effect of stress was found to be more compared to 75% shade. Under open, along with water and salinity stress photo oxidative stress also took place. These stress made earlier induction of resistance mechanism and resulted and more stability of membrane compared to 75% shade (Lambers *et al.,* 1998).

In all the levels of shade, water stress and salinity there was reduction in the membrane stability index compared to control. This reduction is mainly due to the production of the free radicals during stress, which damaged the membrane (John, 1993).

5.2 EFFECT OF ABIOTIC STRESS ANDROGRAPHOLIDE CONTENT.

The effect of shade on andrographolide content of Andrographis paniculata was significant. In open condition, andrographolide content was high. It showed a decreasing trend when shade intensity increased from open to 75% shade level. This increase of andrographolide content under open condition was to acclimate strongly of excess light absorption. The photo reduction of oxygen in the chloroplast increases with increasing levels to excess light absorbed and that plant acclimate to this potential stress by increasing the content of secondary metabolites by the activity of antioxidant scavenging system (Logan 1996).

Water stress increased the andrographolide content. The maximum was recorded 0G3S3W2 (1.35) and OG1S3W2 (1.25). The salinity also increased the andrographolide content. The secondary metabolite andrographolide is reported to have antioxidant activity also and thus the plants under salinity and water stress accumulated more andrographolide to protect them from the salt induced stress, water stress and oxidative stress (Briskin, 2000). Bandeoghu *et al.* (2004) have reported that NaCl stress induced antioxidant responses.

Stomatal conductance and stomatal frequency varied significantly due to different shade levels. The highest stomatal conductance was recorded under open

condition. It was decreased in 50% shade and was slightly increased in 75 % shade. Since the stomatal opening is regulating by light which is immediately by the blue light receptor cryptochrome the stomatal conductance was higher under open condition compared to shaded plants.

The reduction of stomatal conductance was corresponding to increase the water stress from 100 to 30% FC. This reduction was due to chemical signal-ABA arriving from the roots that cause stomatal closure and in turn the decrease in stomatal conductance (Else *et al.*, 1996).

Water stress also causes stomatal closure reduced transpiration rate, a deceasing in the water potential of plants tissue, decrease in photosynthesis and growth inhibition and accumulation of oxygen radical scavenging compounds (Yordhan *et al.*, 2003).

Salinity also reduced the stomatal conductance corresponding to the increase in salinity levels from 3 to 6 mmhos/cm. Due to higher salinity, the stomata were closed and further photosynthesis was impaired. The salinity at soil at soil level causes an inhibition of water uptake because of establishment of narrow gradient of water potential between roots and soil solutions. This causes a reduction in water uptake there by reducing the turgor pressure of leaves whicg caused reduction in stomatal opening (Walter, 1995).

5.3 EFFECT OF ABIOTIC STRESS ON BIOCHEMICAL PARAMETERS OF *Andrographis Paniculata*

Based on the andrographolide content five treatments (including four with Maximum andrographolide content and one with minimum content) were selected from each shade levels. These selected fifteen treatments included combinations of water stress, salinity, and growth stages and shade levels. The plants were exposed to respective treatmentsa for period of fifteen days and changes in different biochemical parameters are compared with respective andrographolide content.

The carbohydrate fractions like starch and sugar contents recorded an increasing trend with increase in shade intensity and were low in open condition. However the maximum was recorded under 75 % shade condition. The total starch content was found to be inversely related with the andrographolide content and this is in accordance to the findings of Winkel-shirley (2002) that the secondary metabolite increased in response to high light stress. A possible role for the increased synthesis of secondary metabolites could be to provide a sink for the excess carbon accumulation in the leaves which correspondingly decrease carbon allocation to starch synthesis and sugar synthesis (Lloyd and Zakhleniuk, 2004). The implication of this is that plants have other mechanisms which help them to store the photosynthesis or direct it into other pathways rather than shutting down carbon assimilation (Lloyd and Zakhleniuk, 2004). They have also reported that the accumulation of sugars will be a signal for inducing the secondary metabolic pathways.

In general, water stress decreased the carbohydrates fractions in the selected treatment combinations. The decrease of carbohydrate fractions might be due to the reversible decrease in sucrose phosphate synthase activities (Vassey *et al.*, 1991). Similar to water stress, salinity also decreased the carbohydrate fractions in andrographis. This may be due to salt induced damage to photosynthetic machinery which resulted in reduced photosynthetic rate. This leads to low carbohydrate fractions under saline condition (Taiz and Zeiger, 2003).

The soluble protein content increased with increasing shade intensity. The plants grown under 75% shade recorded maximum protein content and those under open recorded minimum protein content. But the free amino acid content was decreased in shade condition. The reduction of protein fractions in the open condition was due to the high irradiance destruction of photosynthetic proteins and thylakoid structures. Whereas under shade there was no damage and hence the higher soluble protein content (Walter, 1995).

In water stress, the protein content was decreased with increasing levels of stress. The decrease of protein and amino acid content was due to impaired protein metabolism and syntheses. The activity of stress sensitive enzyme nitrate reductase which is a key enzyme involved in the production of amino acid by nitrogen assimilation might have been reduced under water stress and hence the proteins too. The same trend was observed in salinity also. The protein fractions were converted into compatible cytoplasmic osmotic agents and other stress proteins (Walter, 1995).

The phenol content was high in the open condition. It was decreasing with increasing shade intensity. The increased content of phenol under open condition was due to its protective role against high light intensity in open condition (Walter, 1995).

Catalase activity showed significant variation among the different shade levels. Plants under 75% shade levels showed more catalase activity than those under both open and 50% shade conditions. The reduced activity of the catalase under open light was due to photo inactivation by blue light absorbed by the prosthetic heme groups in catalase enzyme. The blue light mediated photo inactivation of catalase is faster in the presence of oxygen and occurs even in moderate light to which plants are adapted (Feirabend and Engel 1997). The catalase activity was increased under water stress (Hertwig *et al.*, 1992). Catalase also increased under salinity and water stress in all shade levels. In general an increase in catalase activity was observed with a corresponding increase in stress levels. Catalase activity was increased for the purpose of scavenging the free radicals produced during the stress.

The peroxidase activity was high under open condition and minimum under 75 % shade condition. It showed a decreasing trend with increasing shade intensity. This high activity of the peroxidase under open condition is due to steady state increase of enzymes at transcript level (Yoshimura *et al.*, 2000). These results indicated the genes of peroxidase are constitutively expressed for

the immediate and efficient detoxification of H_2O_2 under normal and stress conditions. Harinasut *et al.*, 2003 also reported an increased activity of peroxidase in response to salinity suggesting that the increased POX activities reflect the mechanical properties of the cell wall which inturn could be related to the salt adaption process since cell wall properties are known to be modified by salt stress.

SOD activity was highest in open condition and low in 50 % and 75% shade condition. In general, the open plants are under photo oxidative stress which might cause an accumulation of superoxide and hence an increase of the SOD activity compared to those plants grown in shade levels (John, 1993).

Harinast, (2003) also reported an increased SOD activity under salinity. They have reported that the levels of reactive oxygen species including superoxide and hydrogen peroxide are elevated with increased salinity due to the imbalance in the destruction of ROS.

In the selected treatments plants were exposed to stress and hence were under a higher demand for dissipation of excess excitation energy in the thylakoids, which resulted in increased activities of ROS scavenging. Munne-Bosch and Penuels, (2004) have also reported a higher oxidative stress during exposure of plants to a combination of different stress.

The production of oxygen species in light is mainly mediated by the triplet light excitation state of chlorophyll and subsequent single dioxygen formation and by the photoreduction of dioxygen through the photosynthetic elevation transport chain (Feirabend and Engel, 1997). Among the major arsenal of efficient protective mechanisms which help plants to avoid or vapour oxidative damage. SOD belongs to the antioxidative enzymes which are engaged in detoxing superoxide.

It was increased with salinity and water stress situations. This is mainly due to scavenging of free radical along with catalase and peroxidase and thus protecting the organelle from cellular damage (John, 1993)

5.3 EFFECT OF ABIOTIC STRESS ON THE GENE EXPRESSION.OF *Andrographis paniculata*

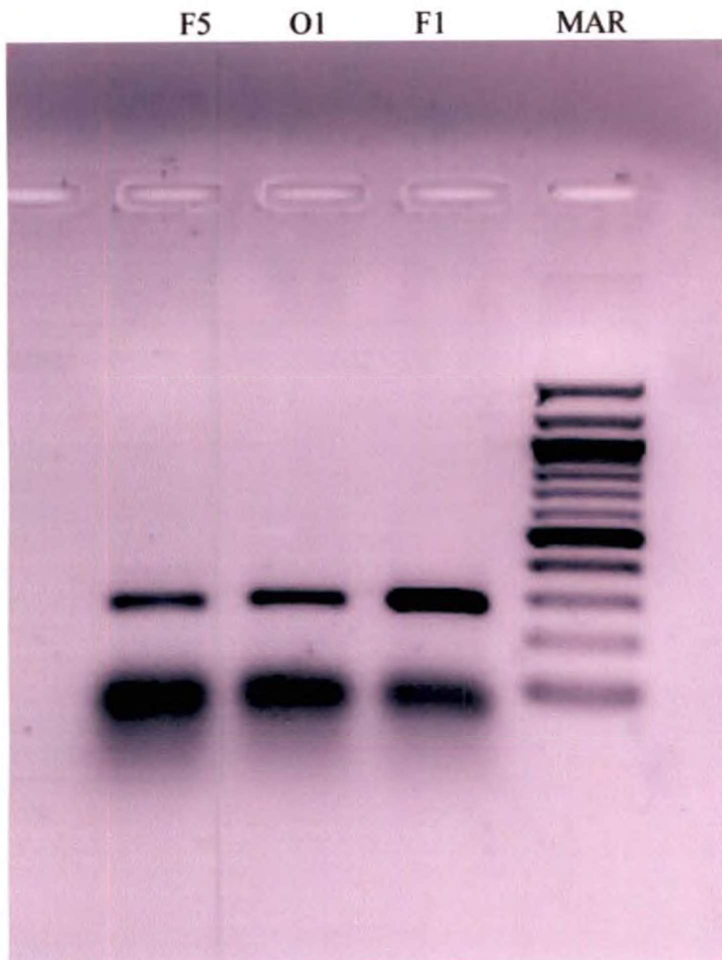
Relative quantitative RT-PCR has been done to study the effect of different stress takes on the level of expression of HMGR. It is an important control point for the synthesis of many of the plant isoprenoids. In plants HMGR is encoded by several gene families and are differently regulated during development, in various tissues and in response to environmental cues (Streamer *et al.*, 1994).

There was significant variation in the expression levels of HMG reductase (HMGR) in all the three selected treatments. The light regulation level of HMGR in was stronger among the treatments. The relative expression of HMGR mRNA was higher under shaded situation compared to open. These results were in accordance with the findings Learned (1996).

Plants regulate HMGR activity at the level of mRNA by differential induction of HMGR gene families. One activity is modulated by light and phytochrome is reported to be the receptor pigment involved in signal transduction (Streamer *et al.*, 1994)

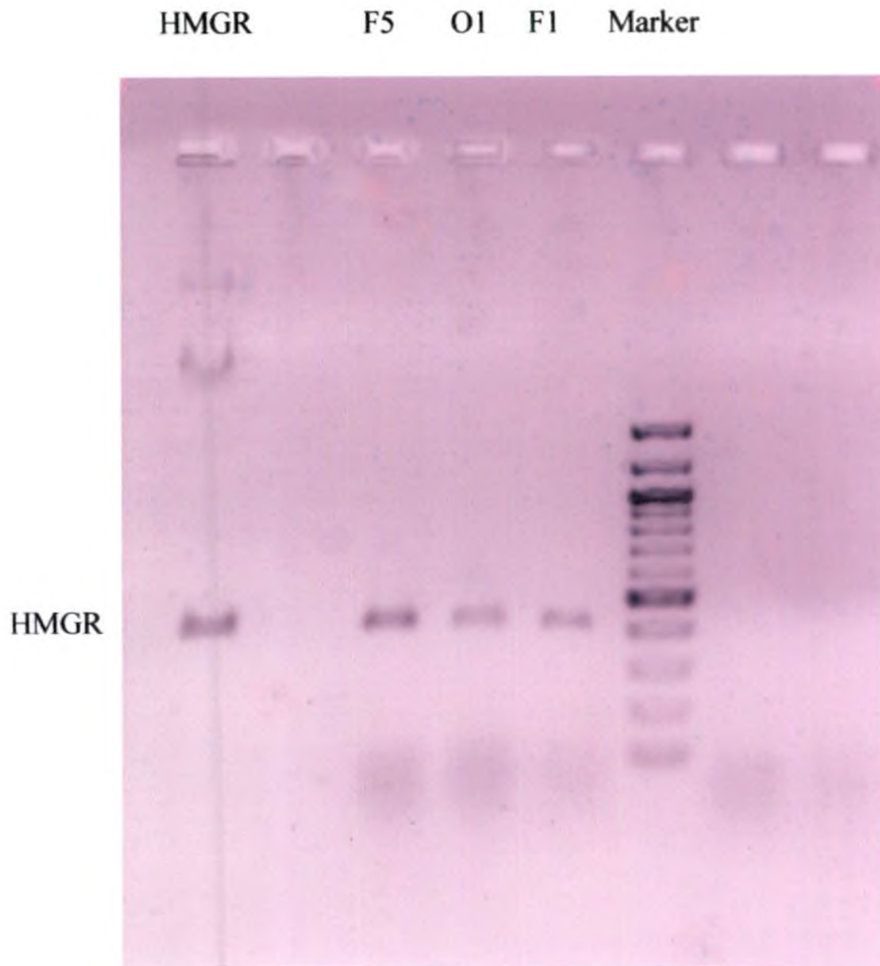
There are contradictory reports regarding the light mediated regulation of HMGR gene expression. Stermer *et al.*,(1994) has reported that red light stimulated the activity of HMGR in plants where as Learned (1996) reported that HMGR mRNA accumulates more under etiolated condition and light repressed the expression of *hmgr*..

In the present study HMGR expression was more under 50% shade when plants were not exposed to water stress or salinity. This result was in accordance with Learned (1996) who also reported an accumulation of HMGR mRNA in etiolated or dark grown plants under continuous irradiance. He has also demonstrated that light responsive expression of HMGR is mediated by cis-acting sequences in the HMG1 promotor and suggested that the regulated accumulation of HMG1 mRNA was a consequence of coordinating promoter activity with



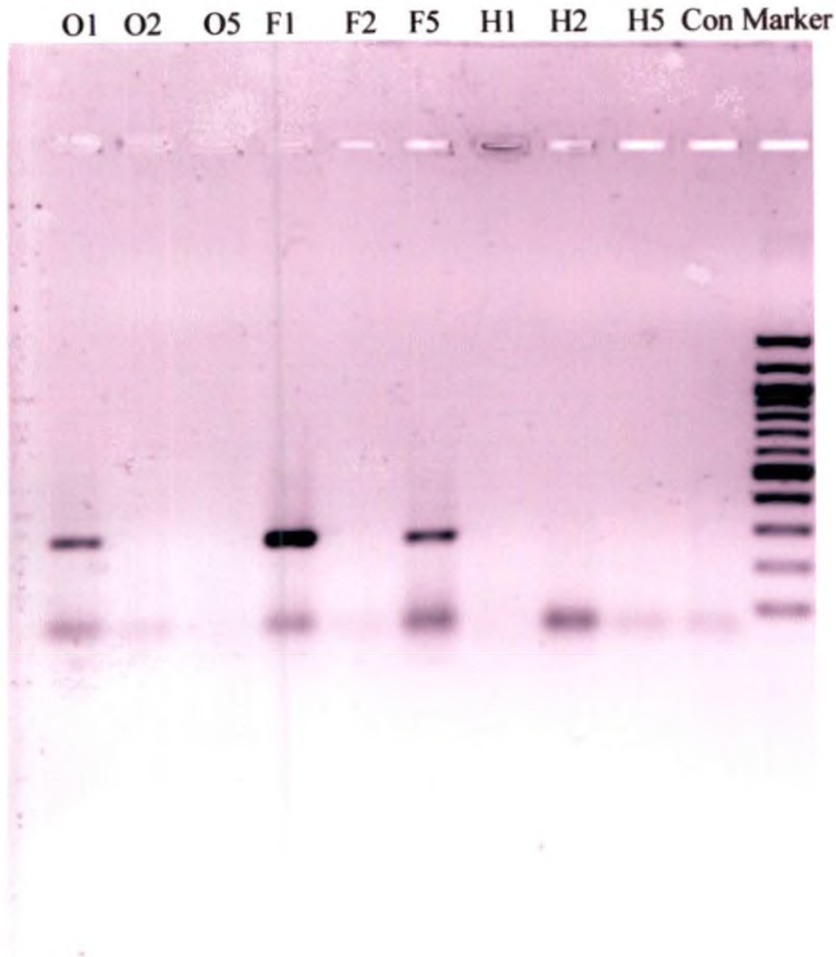
F5 denotes the plant sample which is grown under 50 % shade at 90 DAS with 6 mmhos/cm salinity and 30 % FC, and second line O1 – plants at 60 DAS grown under open condition with combination of water stress (60 % FC) and salinity (6 mmhos/cm) and F1 - plants at 90 DAS grown under 50 % shade condition with salinity (6 mmhos/cm) and with water stress 30 % FC.

Plate 4. Expression pattern of positive internal control actin of CDNA of *Andrographis Paniculata*



F5 denotes the plant sample which is grown under 50 % shade at 90 DAS with 6 mmhos/cm salinity and 30 % FC, and second line O1 – plants at 60 DAS grown under open condition with combination of water stress (60 % FC) and salinity (6 mmhos/cm) and F1 - plants at 90 DAS grown under 50 % shade condition with salinity (6 mmhos/cm) and with water stress 30 % FC.

Plate 5. Effect of abiotic stress on HMGR gene expression of *Andrographis paniculata* relative quantity of RT-PCR of HMGR



Treatment No.	Light level	Salinity (mmhos/cm)	Water stress (FC)	Growth stage (days of planting)
O1	Open condition	6	60%	60
O2	Open condition	6	60%	120
O5	Open condition	3	100%	60
F1	50 % shade	6	30%	90
F2	50 % shade	6	60%	90
F5	50 % shade	3	60%	120
H1	75 % shade	3	60%	60
H2	75 % shade	3	60%	60
H5	75 % shade	3	60%	120

Plate 6. Abiotic stress induced alteration of mRNA levels from selected treatments of *Andrographis paniculata* isolated for RT-PCR of HMGR

features of the light environment.

The expression of HMGR was slightly when plants were exposed to combination of water stress and salinity and the HMGR expression was not related with andrographolide content. RNA blot analysis showed that specific HMGR gene was strongly induced by specific stress or specific signal (Streamer *et al.,* 1994).

Expression pattern of positive internal control (β actin) on cDNA of *Andrographis paniculata* is given in plate 4, effect of abiotic stress on HMGR gene expression of *Andrographis paniculata* relative quantification of RT-PCR is given in plate 5 and 6 show abiotic stress induced alternation of mRNA levels in selected treatments of *Andrographis paniculata* (isolated for RT-PCR of HMGR).

Summary

Relationship between the Andrographolide Content with Biochemical Parameters

The starch content was high in 75% shade condition and low in open condition. The starch content was decreased by salinity and water stress in all shade conditions.

The reducing sugar was high in 75% shade condition and low in open condition. The water and salinity decreased the reducing sugar content in all shade levels and growth stages.

The total free amino acid was high in 75% shade condition and low in 50% shade condition. The salinity and water stress decreased the free amino acid content in all shade conditions.

The protein content was high in 75%shade condition followed by 50% shade condition and open. It was decreased by salinity and water stress in all shade and growth stages.

The phenol content was high in open condition and low in 75% shade condition. The water stress and salinity stress reduced the phenol content in all shade condition and growth stages.

Relationship between the Andrographolide Content Antioxidative Enzymes

Among antixodative enzymes activities, the catalase activity was high in 75% shade level and low in open condition. It was increased in salinity and water stress conditions and all growth stages. The peroxidase activity was high in open condition and low in 75% shade condition. It was increased by salinity and water stress in all shade and growth stages. The SOD also increased in open condition but it was decreased in shade condition. It was low in 75% shade condition. It was also increased by salinity and water stress conditions.

There was an inverse relationship between the sugar, starch and protein content with andrographolide content. There was positive relationship between the catalase activity and andrographolide content in open condition but was positive in the 50 % and 75% of shade. The relationship between phenol, peroxidase activity and andrographolide content was positive. From this experiment it was

concluded that abiotic stress increased the secondary metabolite-andrographolide content which has reported to have an antioxidant activity.

Effect of Abiotic Stress on Gene Expression

The expression level of the key enzymes of the classical mevalonate pathway by which isopenoids are biosynthesized the HMGR was analysed by selective quantity RT-PCR from selected treatments. The expression levels were significant between treatments. The expression levels were significant between treatments. Maximum expression of HMGR mRNA was recorded in plants grown at 50% shade without any stress. The experience was low in plants grown under open condition. In this experiment the over expression of HMGR was not directly correlated with andrographolide content. It was connected that the plastic DOX/MEP pathway might be responsible for the accumulation of higher andrographolide under open condition.

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* Original not seen

Appendix

APPENDIX – I

Weather data prevailed during the cropping period

Year and Month	Maximum temperature	Minimum temperature	Total rainfall (mm)	Total day length (hours)	Relative humidity
2003					
August	30.9	24.3	100.5	6.0	80.8
September	31.4	23.9	10.3	9.1	76.5
October	30.4	23.6	515.9	5.8	84.0
November	30.5	23.2	169	4.5	82.5
December	31.2	21.6	-	8.2	77.1
2004					
January	31.5	21.7	6.8	9.0	76.9
February	32.2	22.1	0.4	9.4	75.3
March	33.1	24.1	1.2	8.8	76.7
April	33.3	24.9	126	8.0	78.3
May	31.0	23.9	447.6	5.3	83.1

EFFECT OF ABIOTIC STRESS ON THE PHYSIOLOGY AND ANDROGRAPHOLIDE CONTENT IN *Andrographis paniculata* Nees.

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Abstract of the
thesis submitted in partial fulfillment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

2004

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ABSTRACT

An experiment was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani to study the influence of abiotic stress on growth, physiological and biochemical parameters and andrographolide content of the medicinal plant, *Andrographis paniculata.* The abiotic stresses include three levels of light (open, 50% and 75% shade); three levels of salinity (control, 3 and 6 mmhos/cm) and three levels of water stress (100%, 60% and 30% field capacity). The treatments were imposed for a period of 30, 60 and 90 days after planting. Growth parameters of plant height, leaf area, leaf area duration, dry matter production, specific leaf area, net assimilation rate and relative growth rate were recorded in plants from different treatment combinations. The treatments having maximum amount of the andrographolide content were selected from each shade level for the detailed study of various physiological and biochemical parameters. The effect of stress on the level of expression of HMG-CoA reductase was assessed by Relative quantitative RT-PCR. The secondary metabolite andrographolide content was estimated spectrophotometrically. The andrographolide content was high (1.35 %) in open condition at growth stage of 90 days with 6 dsm^{-1} salinity and water stress of 60 % FC. Based on the andrographolide content, five treatment combinations were selected from each shade level for further analysis. The physiological parameters like chlorophyll content, stomatal conductance, stomatal frequency and membrane stability index were recorded. The biochemical analyses on starch, sugar, amino acids, protein, phenol, and ascorbic acid were recorded from the selected treatment combinations. The activity of oxidative free radical scavenging enzymes namely catalase, peroxidase, superoxide dismutase were also estimated in the above selected treatment. It was seen that on plant height, leaf area, leaf area duration, specific leaf area, net assimilation rate were high in 50% shade condition. The membrane stability was found to follow trend similar in open and 50% shade condition but low in 75% shade condition. The chlorophyll content was found to

increase under shade condition. The chlorophyll 'a' and total chlorophyll were high in 50% shade and chlorophyll 'b' was high in 75% shade condition. The stomatal conductance and frequency were high in open condition and low in 75% shade condition. The starch, reducing sugar, and protein were high in shade condition. The phenol and total free amino acids were high in open condition and low in 75%shade condition. A positive relationship was obtained between phenol and andrographolide content. The antioxidant enzymes, catalase was high in 75% shade condition and low in open condition but the peroxidase and SOD was high in open condition and low in the 75% shade condition. On the other hand an inverse trend between the sugar, starch and protein content with andrographolide was observed. The water stress and salinity reduce the growth parameters corresponding to the stress levels. The biochemical parameters like starch, sugar, phenol, protein, amino acids showed a reducing trend with increase in the severity while the activity of free radical enzymes showed an increase. There was positive relationship with the catalase activity and andrographolide content. The secondary metabolite under open condition increased under open condition and decreased under the shade condition. From this experiment it was evident that abiotic stress invariably increased the secondary metabolite-andrographolide content, which has reported to have an antioxidant activity. The gene expression was made under 50% shade and there was light inhibition of expression under open condition. The mRNA was found to be reduced under water stress and salinity. It was inferred that the andrographolide accumulation might be corresponding with the activity of plastidic DOXP/MEP pathway.