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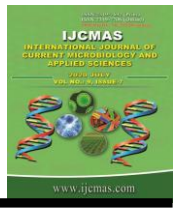

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Performance of *Pongamia pinnata* ROXB. under Waterlogging, Salinity and their Combination in Nursery Environment

Shephali Sachan[1], Sandeep Kumar[2], Pooja Kattiparambil[3] and Anil Kumar[4*]

[1]Tropical Forest Research Institute, Jabalpur-482021 (M.P.), India
[2]Forest Research Institute, Dehradun, - 248006 (Uttrakhand), India
[3]Arid Forest Research Institute, Jodhpur- 342005 (Rajasthan), India
[4]ICAR-Indian Agricultural Research Institute, New Delhi-110012, India

**Corresponding author*

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A B S T R A C T

The world is facing severe and unpredictable challenges due to increasing abiotic stresses as a consequence of the earth's changing climatic conditions. The increasing waterlogging and salinity stress problems are the example of such disturbances caused in nature. In the present study, the effect of waterlogging, salinity and combined waterlogging & salinity stress on morphological, physiological and biochemical parameters along with protein profile of *Pongamia pinnata* at seedling stage under nursery conditions have been discussed. Pot culture experiments were conducted in factorial RBD design to observe the effect of waterlogging (W), salinity (S) and combined salinity & waterlogging (SW) stress on the selected seedling under nursery conditions for one year. Waterlogging stress condition was created by perforating the polybag at specific height with standard size and watered daily. Salinity was maintained at 8 dS/m salt concentration. The amount of water equal to the calculated field capacity was provided to each polybag. Total biomass and leaf area were measured in morphological parameters. Physiological parameters *viz.* photosynthetic rate and stomatal conductance of the seedlings were measured. Total chlorophyll, proline and protein content were estimated for biochemical analysis. Protein profiling of seedlings was performed by SDS-PAGE method. The outcome of the experiment showed that salinity treatment had major negative impact on biomass which can also observe in leaf area. Under waterlogging treatment, the seedling showed very appreciative avoidance and adapted behavior. The seedlings under salt + waterlogging managed to tolerate the combined stress condition which was opposite to deleterious impact of combined abiotic stresses. Two new bands observed under 8 dS/m S + W shows the involvement of protein in the tolerance behavior of seedlings. The plantations of suitable tree species in such areas will be helpful in sustainable forest management and

Introduction

The world is facing severe and unpredictable challenges due to increasing abiotic stresses as a consequence of the earth's changing climatic conditions[9]. The increasing waterlogging and salinity stress problems are the example of such disturbances caused in nature.

Waterlogging/flooding stress refers to a condition when water is present in excess amount than its optimum requirement. The soil pores which are previously occupied by oxygen gets filled with excess water causing oxygen deficiency[47]. While, accumulation of dissolved salts in the soil water in a higher amount than required causes salinity problems, inhibiting the plant growth[39].

Waterlogging induces many alterations in soil physico-chemical properties like pH, redox potential and oxygen level, hence the plants growing on waterlogged soil face the stressful environment in terms of hypoxia (deficiency of O_2) or anoxia (absence of O_2) condition[5]. Approximately 10% of all irrigated farmland comes under frequent waterlogging areas worldwide[38]. The effect of waterlogging in large, flat areas for several days creates the condition of flood causing damage to trees and forests[36]. The waterlogged and marshy lands cover 0.52% of India's total land area[39]. The districts located in Gangetic Plains, the Brahmaputra Valley, Eastern Coastal plain and in western Rajasthan (Indira Gandhi Canal command area) are under the strong pressure of waterlogging. About 202 districts of the country are affected by waterlogging, the severity of which range between 6-15 per cent in Bihar, Odisha and Assam[39].

Salinity is also the major abiotic stress which can brutally limit the crop production, especially in arid and semi-arid regions. The substantial increase in soil salinity could affect the long-term health, productivity and survival of trees, and potentially leave discharge sites further degraded[28]. More than 45 million hectares (mha) of irrigated land which accounts to 20% of total land has been damaged by salt worldwide and 1.5 m ha is being out of use each year due to high salinity levels in the soil[42]. The salts affect the plants in two ways *i.e.*, osmotic potential and ionic toxicity. Under salt stress, the osmotic potential becomes higher in the soil solution as compare to plant cells as the concentration of salt is high in the surrounding, due to which plants ability to take up water and other essential nutrients gets decline[38]. In India, about 300 million hectares of coastal land is lying barren and uncultivable because of soil affected by salinity[13]. It was estimated that 142 districts distributed in Gujarat, Rajasthan, Punjab, Haryana, Uttar Pradesh, Karnataka, Andhra Pradesh and Tamil Nadu are affected by soil salinity or alkalinity[39].

About 60-80 million hectares of land is affected to some extent by combined salinity and waterlogging stress[17]. In the irrigated areas of semi-arid regions, especially in northwest India, a considerable recharge to the groundwater leads to waterlogging and secondary salinization. Soil salinization is the process of accumulation of excess salts in the root zone creating osmotic stress, which results into physiological drought stress combining with ionic toxicity, whereas waterlogging is the presence of excess water in the root zone of plants resulting in poor gas exchange and eventually anaerobic conditions. Because waterlogging or salinity alone is harmful to the growth of most dryland species, their combined effects would be expected to be especially damaging[32].

The selected species *Pongamia pinnata* (Family – Fabaceae), popularly known as Karanj, is a medium size shade tree species[30] widely distributed in the Indian subcontinent,

south-east Asia, Fiji, Myanmar, northern Australia, the East-African coast, southern China and the Seychelles Islands.

In India, this tree is distributed throughout the country except for temperate regions and is considered to be native to the western ghats of India[12]. It is a preferred nitrogen-fixing species for controlling soil erosion and binding sand dunes because of its dense network of lateral roots[4] and phytoremediation quality[30]. The species is famous for its seed oil which has biodiesel potential, many industrial and medicinal applications[4].

The present study focuses on the species morphological, physiological, biochemical and molecular response against artificially applied waterlogging, salinity and combined waterlogging + salt stress conditions. The study will be very helpful to know the potential of *Pongamia pinnata* species against the selected abiotic stresses in the nursery environment.

Materials and Methods

Experimental site and plant material

Pot culture experiments were conducted in the nursery of Tropical Forest Research Institute (TFRI), Jabalpur (M.P.) for one year.

The location of experimental site in the nursery was specified as $23^0$5'57.2" N latitude, $79^0$59'2" E longitude and 394 m altitude above the sea level according to Global Positioning System (GPS). *Pongamia pinnata* (Karanj) tree species was selected for the study. The seeds were collected from TFRI campus during March to May and sown in the nursery beds in June, just before first shower of rainfall, which is favourable for normal and healthy germination process.

Growth condition and experimental design

The size of nursery mother beds was fixed to be 10 m x 1 m and the sowing medium was sand which provided sterile conditions for germination of seeds. Germination period for the *Pongamia pinnata* was observed to be 5-7 days. After the germination of seeds, the seedlings having 2-3 leaves were transferred to transparent polythene bags of standard size (15 cm x 23 cm) filled with soil, sand and farm yard manure (FYM) in 2:1:1 ratio. The polythene bags were initially placed under shade for one month to protect the seedlings from direct sunlight and then kept in open area for another one month in order to acclimatize them with the prevailing conditions.

Factorial Randomized Block Design (RBD) was adopted to conduct pot culture experiments in order to observe the effects of waterlogging, salinity and their combination stress.

After acclimatizing seedlings in polythene bags for a month in open areas, three treatments (W- Waterlogging, S – Salinity and SW – Salinity and Waterlogging) were provided to the seedlings including control (C). Each treatment consisted of nine seedlings and the experiment was replicated thrice.

Waterlogging treatment

Waterlogging experiments in the seedlings planted in polybags under nursery condition were conducted according to the experimental design mentioned above. The amount of water equal to the field capacity, 24.86% [Soil moisture at field capacity (%) = (WW-DW) x 100/DW, where, WW - Wet Weight of soil + plant (g) and DW - Dry Weight of soil + plant (g), adopted by Tyree[52] *et al.*, (2002)] was given to each polybags.

Artificial waterlogging conditions were created by making 8 holes of size 6±0.5 mm in each polybag through punching machine in the periphery of the polybags below 3 cm from surface[33]. The polybags were watered daily with the amount equal to the field capacity[21,14]. In the control plants, the holes were made at the bottom of polybags and watered at the interval of 2-4 days. This way no waterlogging was observed in control plants as the excess water drained out of the holes from the bottom[25]. Moreover, waterlogging was attained in polybags perforated at 3 cm from the surface.

Salinity treatment

Salinity was artificially created in the polybags through supply of salt (NaCl) dissolved water, which was maintained to be 8 dS/m after regularly measuring the electrical conductivity of the soil[44].

Salinity and waterlogging combined treatment

Combined salinity and waterlogging conditions were artificially created by adding NaCl equal to 8 dS/m through irrigated water to polybags equivalent to the calculated field capacity daily.

Morphological parameters

Total biomass of each seedlings was estimated by destructive method at the end of experiment. Mature leaves were plucked and size was measured using Systronics make Leaf Area Meter at the end of experiment. Three leaves were considered per treatment and replicated thrice. The average readings were considered for statistical analysis.

Physiological parameters

Physiological parameters *viz.* photosynthetic rate and stomatal conductance of the seedlings of selected species were measured at the end of one year between 8:00 AM to 10:00 AM using CID-340 make Photosynthetic System. During measurement of physiological parameters, the Photosynthetically Active Radiation (PAR) ranged from 803-2298 µmol/m^2/s were also noted. Calibration of the instrument was done before use. After switching on the instrument and feeding required parameters, it was kept for 30 minutes for stabilization. Mature leaves were placed in the leaf chamber of the instrument. Three leaves taken per treatment and replicated thrice[26].

Biochemical parameters

Biochemical parameters like chlorophyll, proline and protein content were estimated at the end of experiment. Chlorophyll was estimated following Arnon's[3] method (1949). Weighed 0.1 g of fresh leaf sample, finely cut and well crushed to fine pulp in pestle mortar with the addition of 5 ml of 80% acetone. Centrifuged at 5000 rpm for 5 min and transferred the supernatant in a test tube. Vortexed the residue with the addition of 5 ml of 80% acetone and again centrifuged it for 5 min in 5000 rpm. The supernatant was collected in the same test tube. Mixed and read the absorbance of the solution at 645, 663 and 470 nm against the solvent (80% acetone) blank.

Calculation

The amount of extracted chlorophyll was calculated in the mg chlorophyll/g tissue by following formula:

Chlorophyll a (mg g-1) = [(12.7 × A663) - (2.6 × A645)] × V/1000 x W

Chlorophyll b (mg g-1) = [(22.9 × A645) - (4.68 × A663)] × V/1000 x W

Total Chlorophyll = [(20.2× A645) + (8.02× A663)] × V/1000 x W

Proline content in the leaves was quantified by the spectrophotometric method followed by Bates[8] *et al.*, (1973). Extract 0.1 g of dried leaf sample by homogenizing in 5 ml of 3% aqueous sulphosalicyclic acid. Centrifuged at 3000 rpm for 25 min and homogenate was filtered through Whatman No. 2 filter paper. 2 ml of filterate, glacial acetic acid and acid ninhydrin were taken in a test tube and heated it in the boiling water bath for 1 hour. Terminated the reaction by placing the tube in ice bath. Added 4 ml toluene to the reaction mixture and stirred well for 20-30 seconds. Separated the toluene layer and warmed to room temperature. Measured the red colour intensity at 520 nm. Ran a series of standard with pure proline in a similar way and prepare a standard curve. Toluene was taken as a blank for both sample and standard. Found out the amount of proline in the test sample from the standard curve.

Calculation

The proline content was expressed as follows:

$$\mu \text{ moles per g tissue} = \frac{\mu g \text{ proline/ml} \times \text{ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

Where, 115.5 is the molecular weight of proline.

Protein content was estimated by spectrophotometric method followed by Bradford[10] (1976). 50 µg of fresh leaf sample was thoroughly grounded to a fine powder in a pre-chilled mortar by liquid nitrogen and then mixed with 5 ml lysis buffer (1.0M Tris Base buffer of pH 8.0 containing 25 mM EDTA, 10 mM β-mercaptoethanol and 1% PVP). Centrifuged it for 10 min in 13000 rpm in 4^0C. Supernatant was used for analysis. 100 µl supernatant taken in a test tube. Prepared standard curve of concentration 10 to 100 µl working standard. Both samples and standards were prepared in duplicates. 100 µl of distilled water was taken into a tube to provide the reagent blank. Made the volume 300 µl in all test tubes with distilled water. Added 3 ml of dye to each tubes and mixed well by shaking manually. Kept the test tubes in dark for 15-30 minutes. Measured the absorbance at 595 nm. The concentration of protein in the samples were calculated through standard curve.

Protein profile

Protein profiling of fresh leaves was done after one year experiment by 'Laemmli[31] Method' using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS – PAGE). The protein of fresh leaves was isolated by following Bradford's method and then proceeded for profiling.

Reagents required

Acrylamide/Bis-acrylamide solution (30% stock) – 30 g acrylamide and 0.8 g bis-acrylamide was dissolved in 60 ml distilled water and made the volume upto 100 ml. Stored at 4^0C.

Ammonium Persulphate (10%) – 10 g ammonium persulphate was dissolved in 100 ml distilled water and stored as 1 ml aliquots in microcentrifuge tubes at -20°C.

Tetramethylethylenediamine (TEMED)

SDS (10%) – 10 g SDS was dissolved in 100 ml distilled water, heated till bubbles emerge and stored at RT.

Separating gel Buffer (0.98M, pH 8.8) – 11.87 g Tris Base was dissolved in 100 ml distilled water. Adjust the pH to 8.8.

Stacking gel Buffer (0.325M, pH 6.8) – 3.94

g Tris Base was dissolved in 100 ml distilled water. Adjust the pH to 6.8.

Electrode/Tank Buffer (10 X) –
Tris Base (50 mM) – 18.15 g
Glycine (384 mM) – 86.25 g
SDS (0.1%) – 3 g

Made the volume upto 3000 ml and heated on hot plate till bubbles appear, cool and store at RT.

Destaining solution I (DS I – 1000 ml)
Methanol – 500 ml
Glacial Acetic Acid – 100 ml
Distilled water – 400 ml

Destaining solution II (DS II – 1000 ml) –
Methanol – 300 ml
Glacial Acetic Acid – 100 ml
Distilled water – 600 ml

Staining solution (1000 ml) –
Coomassie Brilliant Blue (R250) – 2.0 g
Methanol – 400 ml
Glacial Acetic Acid – 200 ml
Distilled water – 400 ml

Table.1 Composition of Separating gel (12%)

Composition	24.46 ml
30% Acrylamide/Bis – acrylamide	9.6 ml
Separating Buffer (pH 8.8)	12 ml
Distilled water	2.4 ml
SDS (10%)	240 µl
TEMED	20 µl
Ammonium Persulphate (10%)	200 µl

Composition of Stacking gel (5%)

Composition	7.50 ml
30% Acrylamide/Bis – acrylamide	2.0 ml
Stacking Buffer (pH 6.8)	3.2 ml
Distilled water	2.12 ml
SDS (10%)	66 µl
TEMED	10 µl
Ammonium Persulphate (10%)	100 µl

Composition of Sample buffer (pH 6.8)

Composition	100 ml
Tris Base (625 mM)	7.57 g
SDS (2%)	2 g
Glycerol (10%)	10 ml
β – mercaptoethanol (5%)	5 ml
Bromophenol blue (0.001%)	0.001 g

Procedure

Assembled the components SCIE-PLAS TV400Y SDS – PAGE unit: tall glass plate, notched glass plate, 1 mm spacers, gel casting frame, gel casting stand, teflon comb, electrophoresis chamber, electrode wires and voltage

unit.

The glass plates were assembled using clamps and 1 mm spacers.

The solution for 12% separating gel was prepared and poured between glass plates using a gradient mixer with constant stirring, leaving a gap of 3.5 cm from the top. Polymerization of gel took place 20-30 minutes.

Now after separating gel polymerization, teflon comb was inserted and 5% stacking gel was prepared. The gel was poured carefully into cavity from sides using micropipette to prevent formation of bubbles till top of the notched plate. Polymerization of gel took place 10-15 minutes.

Meanwhile prepared the loading dye, for running standard (Bovine serum albumin/protein ladder – Low range protein marker, 3-40 kDa and Mid range protein marker, 14-80 kDa) took sample buffer and standard in 1:1 ratio (10 µl each). For running sample whatever the concentration was got from spectrophotometer readings for 100 µl is taken in 1:1 ratio with sample buffer.

The standard and samples loading dye were then kept for 10 min in 100^0C in water bath. After warming cooled down at room temperature and ready for loading.

After polymerization of stacking gel, the comb was removed carefully out of the stacking gel.

The sandwich assembly (glass plates with frame) seperated from gel casting stand and inserted in the electrophoresis chamber.

The electrode running buffer was poured in the chamber upto the top of the glass plates such that wells and thin electrode wires were fully covered.

A pre run was carried out at 60 V for 15-30 minutes to flush all ions out from the wells to decrease interference with sample run.

Now 20 µl of loading dye of each (standard and samples) were loaded into wells carefully with the help of micropipette.

Plugged in the electrodes red to red and black to black on voltage unit and electrophoresis chamber. The electrophoresis was carried out at 110V during stacking and continued at 220 V for 8-9 hours till the dye front reached the bottom of the gel.

After completion of the gel run the sandwich assembly was removed from the electrophoresis unit.

The buffer in the assembly was discarded and the glass plates were separated using plastic spatula after removal of spacers in the tray full of distilled water. The gel got separated.

The gel was then transferred to DS I and kept for overnight with gentle shaking.

After discarding DS I, the gel was incubated in staining solution of Coomassie Brilliant Blue for 20 minutes with gentle shaking on shaker platform.

After staining, the excess stain from the container was collected for future use and the gels as well as the container were rinsed with small quantity of DS II followed by further incubation in fresh DS II for few hours to effectively destain the gel.

The washing step in DS II was continued till the gel became clear of any background stain.

The protein bands appeared after DS II in the gel was properly scanned by Gene Sys scanner.

The molecular weight of the sample band protein were determined by Gel Analyzer software.

Statistical analysis

Analysis of Variance (ANOVA) and

significant variation among different treatments was observed by comparing calculated F values with tabulated F values. Interactions among different treatments were also calculated using factorial RBD design. Pairwise comparison among the selected treatments was done after calculating critical difference (CD) at 5% significance levels.

Results and Discussion

Morphological parameters

Significant (P<0.05) difference in total biomass and leaf area was observed in *P. pinnata* tree species seedlings under control, waterlogging, salinity and combined salinity and waterlogging treatments at the end of experiment (Table 1).

The total biomass was found to be maximum (16.03 g) under waterlogging and minimum (1.89 g) under salinity treatment respectively. Leaf area of *P. pinnata* leaves was found maximum (43.57 cm^2) under waterlogging and minimum (11.80 cm^2) under control treatment. The leaves were very tiny for measurement under salinity treatment (Fig. 1).

Physiological parameters

Significant (P<0.05) difference in photosynthetic rate and stomatal conductance was observed in *P. pinnata* tree species seedlings under control, waterlogging, salinity and combined salinity and waterlogging treatments at the end of experiment (Table 1). The photosynthetic rate and stomatal conductance were found maximum (3.07 µmol/m^2/s and 36.31 mmol/m^2/s) under waterlogging and minimum (1.22 µmol/m^2/s and 3.36 mmol/m^2/s) under salinity + waterlogging treatment respectively. The leaves were very small and rare, unfit for leaf chamber for measuring physiological parameters under salinity treatment (Fig. 2).

Biochemical parameters

Significant (P<0.05) difference in proline and protein content was observed while non - significant (P>0.05) difference in total chlorophyll content was obtained in *P. pinnata* tree species seedlings under control, waterlogging, salinity and combined salinity and waterlogging treatments at the end of experiment (Table 1).

The total chlorophyll was found maximum (33%) under control and waterlogging treatment equally while minimum (15%) under salinity + waterlogging treatment respectively. Proline was found maximum (55%) under salinity + waterlogging treatment while minimum (3%) under control treatment seedlings respectively. Protein was found maximum (46%) in control treatment and decreased under stress treated seedlings. The minimum protein (13%) was observed under salinity treated seedlings (Fig. 3).

Protein profiling

In *P. pinnata*, nine bands were showed by control at 77.70, 54.43, 42.34, 36.91, 30.41, 23.94, 18.92 and 17.41 kDa. No bands were observed in individual waterlogging and salinity experiment. In 8 dS/m + SW two new bands were observed at 47.26 and 32.84 kDa along with other bands. The intensity of bands were high in control followed by 8 dS/m + SW (Fig. 4).

Morphological parameters

The *P. pinnata* seedlings shown maximum biomass and leaf area under waterlogging treatment which may be due to the development of some morphological changes like formation of adventitious roots, initiation of hypertrophied lenticels and establishment of aerenchyma[5]. Wang[54] *et al.*, (2016) suggested that under waterlogging and

flooding, the tree species with strong waterlogging tolerance have higher ability to maintain energy-metabolic balance and their growth can be maintained at certain level. Also, increased leaf size could be the strategy to drain more water from the bottom, facilitating higher transpiration from the leaves. This way more water is removed from the soil[1, 29]. The broader leaf size accumulates more and more carbon which increases plant biomass[5]. Shukor[46] *et al.*, (2014) also observed increase in height and biomass in *Azadirachta excelsa* under artificial waterlogging treatment for limited period. The insignificant reduction in the growth attributes of *Hopea odorata* Roxb. tree species under waterlogging treatments, proved to be as tolerant species against short and long-term waterlogging[1]. Su[49] *et al.*, (2011) found higher biomass in transgenic poplar trees under waterlogging stress.

Table.1 Critical difference and standard error (±) values of the selected parameters

Parameters	Critical Difference (CD)	Standard Error (SE ±)
Total biomass	1.994	0.799
Leaf area	5.796	2.324
Photosynthetic rate	0.149	0.060
Stomatal conductance	1.617	0.648
Total chlorophyll	NS*	0.461
Proline	21.033	8.432
Protein	0.565	0.227

*NS = non-significant

Fig.1 Effect of waterlogging, salinity and combined salinity and waterlogging stress on total biomass and leaf area of *Pongamia pinnata* seedlings

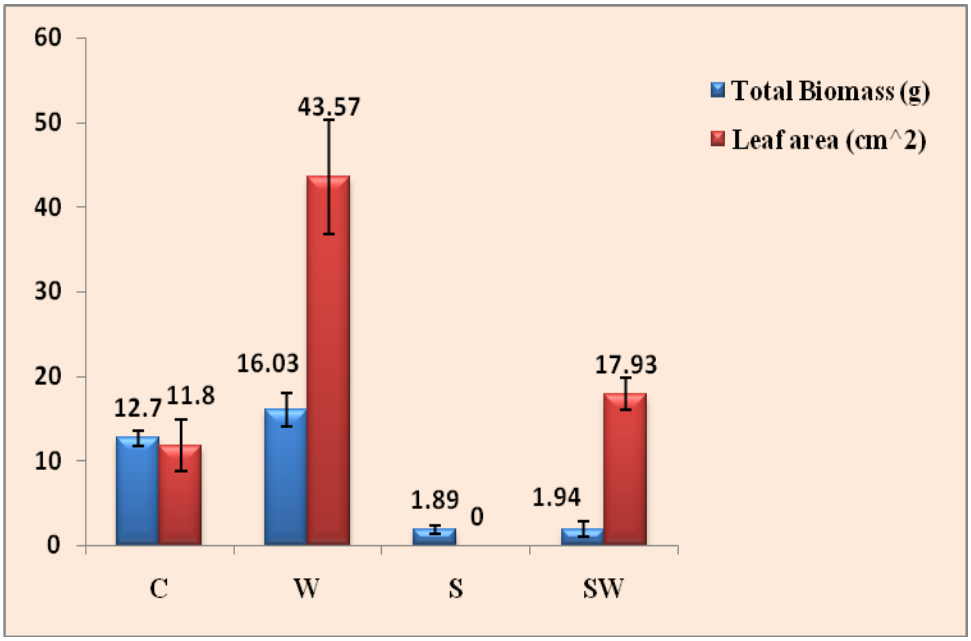
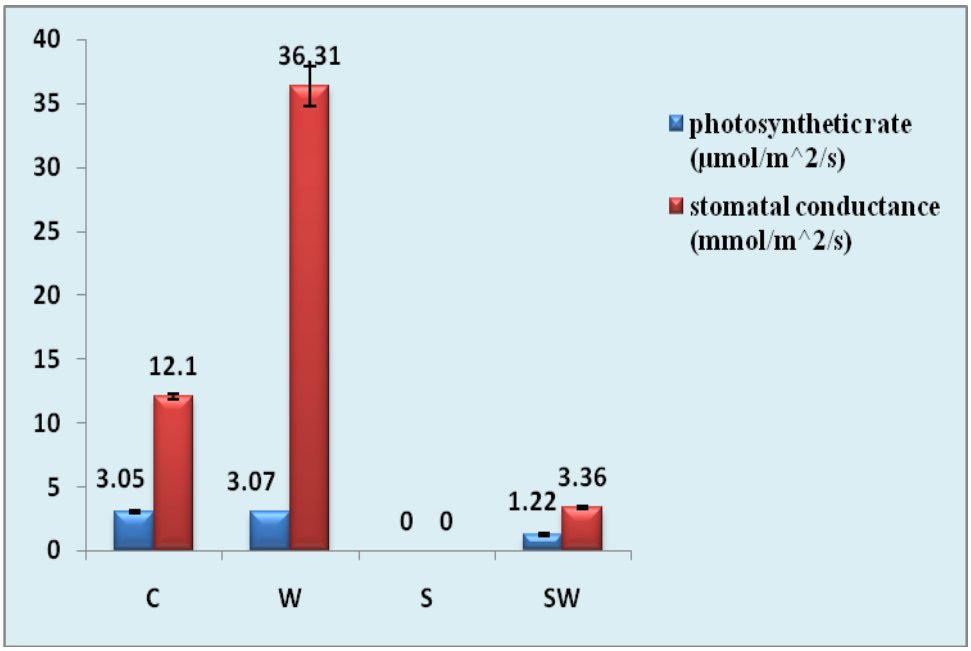
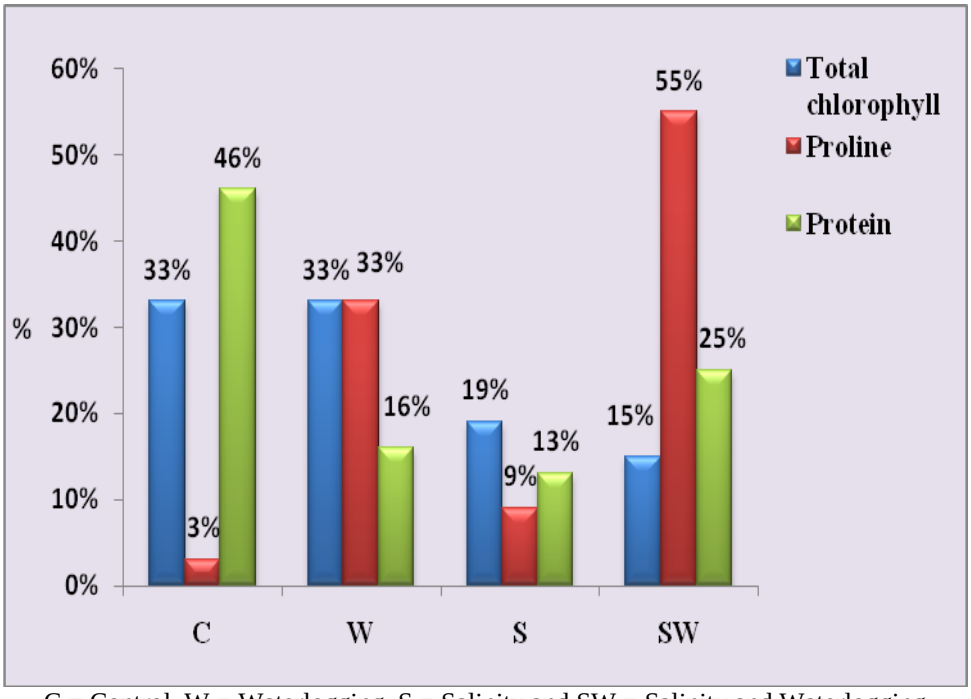


Fig.2 Effect of waterlogging, salinity and combined salinity and waterlogging stress on photosynthetic rate (Pn) and stomatal conductance (C) of *Pongamia pinnata* seedlings



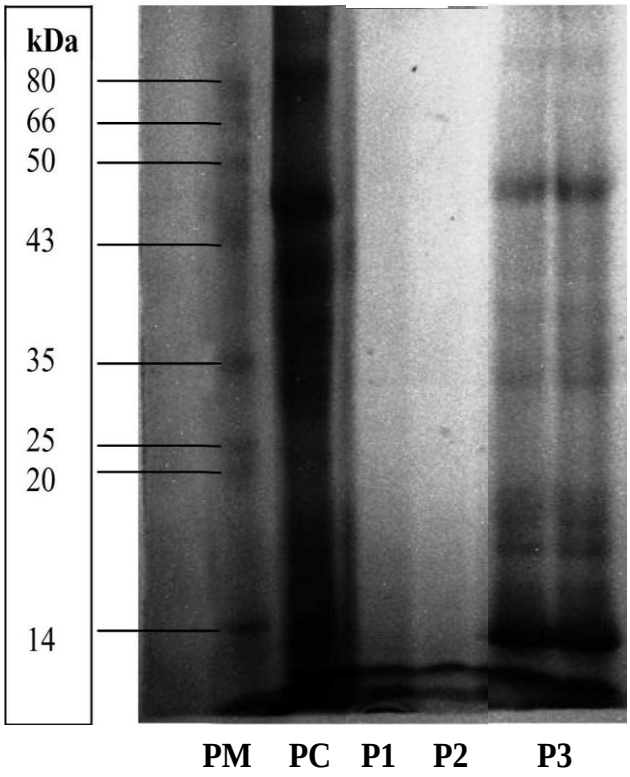
C = Control, W = Waterlogging, S = Salinity and SW = Salinity and Waterlogging

Fig.3 Effect of waterlogging, salinity and combined salinity and waterlogging stress on total chlorophyll and proline of *Pongamia pinnata* seedlings



C = Control, W = Waterlogging, S = Salinity and SW = Salinity and Waterlogging

Fig.4 *P. pinnata* protein profile. PM – Protein Marker (14-80 kDa), PC – Control, P1 – Waterlogging, P2 - 8 dS/m Salinity, P3 – 8 dS/m Salinity + Waterlogging



Total biomass declined with severity of salinity stress. The decrease in biomass is the negative impact of salt due to the increased ionic toxicity and higher osmotic potential[37]. Singh[47] (1999) also observed higher fresh weight and dry biomass in control for *P. pinnata* seedlings, which decreases with increase in salinity. The presence of rare and small leaves unfit for measurement corroborates with Abdul Qados[2] (2011) studies who reported that low concentration of salinity do not show any significant effect, but increase in salt concentration shows deleterious effect on leaf number and size in plant species. The accumulation of NaCl in the cell walls and cytoplasm of the leaves reduces leaf number as the vacuole sap accumulate more salt than required resulting into increase in salt (ionic) concentration leading to their quick death and shedding[37].

The waterlogging and salinity alone is harmful, their combination effects would be expected to be especially damaging[32]. Similar results were also noted by Singh[48] *et al.*, (1996) in *D. latifolia*, which when exposed to various managed water salinity concentrations of 0.3, 0.6 and 0.9 ds m^{-1}, the Above Ground Biomass (AGB) reduced to 29, 50 and 63% respectively. Salinity (12 dS/m) and hypoxia alone as well as in combination caused a significant reduction in shoot biomass of wheat[45]. The slight increase in biomass as compare to individual salinity stress might be due to persistence of leaves till the end of experiment. Against the deleterious effect, the increased leaf size as compare to control could be due to morphological adaptation feature of development of lateral roots more than main tap root, also reported by Gibberd[21] *et al.*, (2001) and Arpiwi[4] (2013) or due to the

phenomena of transporting the excess salt to the vacuole or older tissues thereby protecting the plant from salinity stress[24].

Physiological parameters

The photosynthetic rate and stomatal conductance were found maximum under waterlogging treatment. Jackson and Colmer[27] (2005), observed that certain species are highly productive in waterlogged areas due to some physiological adaptations. The photosynthetic rate and biomass content were found positively and significantly correlated with each other depicting that higher photosynthetic rate accumulates more biomass in the seedlings. Higher photosynthetic rate and stomatal conductance accumulated more biomass by maximising the loss of water through stomatal opening (transpiration), because water is abundantly available under waterlogged conditions.

The presence of rare and tiny leaves shows unability to produce under the salinity treatment till the end of one year of experimentation. Freitas[20] *et al.,* (2017) also observed that *Tectona grandis* in the early stages of development is highly sensitive to salinity stress, slowing down the vegetative growth.

The combination of waterlogging with ionic toxicity caused by salt stress, thus create more drastic situation for plants to survive. Combination of salinity and waterlogging leads to land salinization by transporting salts to the surface and affecting rate of photosynthesis and stomatal conductance in plants[18, 19, 15]. Tounekti[51] *et al.,* (2018) reported that combined stresses decreases stomatal density, gas exchanges due to stomatal closure and inhibits photosynthesis processes. The findings are in line with Azizi[7] *et al.,* (2017), who reported that high concentration of flooding and salinity water leads to strong reductions in photosynthesis and stomatal conductance in *Populus euphratica.*

Biochemical parameters

Chlorophyll content in the leaves of the selected tree species was found non-significant under the various treatments but was found maximum under waterlogging treatment as the content is related with photosynthetic rate. The results are in agreement with the studies conducted by Gratani[23] *et al.,* (1998) in *Quercus ilex* L species who found the positive correlation between chlorophyll content and photosynthetic rate. The chlorophyll followed decreasing trend then from control = waterlogging, salinity to their combination treatment. Parida[40] *et al.,* (2004) described that NaCl has limiting effect on photochemistry, that ultimately affects photosynthesis by inhibiting chlorophyll synthesis. Salinity reduces chlorophyll 'a' chlorophyll 'b' and total chlorophyll contents in agricultural crops[2]. The interaction of salinity and waterlogging has reduced total chlorophyll content in tree seedlings, which corroborates several studies conducted by different authors[18, 19, 15].

The activation of protective system against the generation of reactive oxygen species (ROS) leads to production of number of enzymatic and non-enzymatic antioxidant species[13]. Highest proline content under combination treatment in the present study, supports other studies conducted by Matysik[34] *et al.,* (2002) in *Linum usitatissimum*; Tounekti[51] *et al.,* (2018) in *Salvadora persica* and Carter[11] *et al.,* (2006) in *Casuarina obesa*. Increase in proline content under saline-waterlogged conditions can be attributed to the regulation of foliar Na^+, Cl^- and K^+ concentrations[37] due to which the seedlings managed to respond better than expected from combination of two deleterious abiotic stresses. The upright morphological

and physiological attributes of *P. pinnata* seedlings under waterlogging treatment as compared to control could also involve the role of proline which is second highest here. Increase in proline under stress protects the plant from free-radical induced injury and helps in stabilizing protein and DNA repair thus protecting from oxidative stresses[13]. Parvin and Karmoker[41] (2013) also observed increase in proline content under waterlogging stress in *Corchorus capsularis*. The role of protective enzymatic and non-enzymatic compounds become ineffective when the stress persistence for longer time as there is rise in reactive oxygen species which also leads to destruction in the protective system of plant. This might have happened under salinity treatment.

The protein content was observed at its peak in control treatment and then found to be decreased in the selected abiotic stress treatments. The number of common negative changes observed in a plant cell due to abiotic stress generated unfavourable environment like generation of reactive oxygen species (ROS), downregulation and switch off of certain proteins and destruction and damage of cellular components, as a result of which the total protein amount goes down[55]. Parvin and Karmoker[41] (2013) observed decrease in protein content under waterlogging stress in *Corchorus capsularis*. The lowest amount of protein content under salinity treatment shows the lethal impact of sodium chloride accumulation. Salt stress induces an accumulation of superoxide radicals (O_2) and hydrogen peroxide (H_2O_2) in different cell compartments, including chloroplasts, mitochondria and apoplastic space, which correlates with increases in some oxidative stress parameters, such as lipid peroxidation and protein oxidation[22, 35]. Protein concentration significantly decreased with the increase in salt levels in *Paulownia imperialis* and *P. fortunei* trees compared to controls in which no sodium chloride was added. He concluded that decrease in protein contents is mainly due to loss of K+ ion under salinity stress, which helps in synthesis[6]. During the course of the experiment the salinity and waterlogged stress seedlings showed slight increase in protein content as compare to individual stresses which may be due to synthesis of some different proteins in response to stress[16] which can be seen in the results of protein profiling.

Protein profiling

The control proteins observed in 8 dS/m + SW combination along with two new protein bands. Although, morphological and physiological response were found to be superior in waterlogging experiment than 8dS/m salinity and their combinations, it might be possible in first that control proteins were no longer required and its production became suppressed and in later these proteins required in order to manage the severity. The combination treatment seems to be showed some positive effect as compared to individual stresses which shows plant protective measure for maintaining resistivity in 8dS/m + SW. The response of plants to combinations of two or more stress conditions is unique and cannot be directly extrapolated from the response of plants to each of the different stresses applied individually. Different stress combinations require novel types of defense and acclimation responses[50]. The new protein bands (47.26 and 32.84 kDa) observed may fall under photosynthesis (46.30 kDa), carbohydrate (47.20 kDa) and lipid (32.10 kDa) related proteins[53, 17].

In conclusion, the current elevating waterlogging and salinization situation has imposed the huge damage to agriculture and forestry, giving rise to barren and degraded areas in this constantly growing population period. In the present study, the various changes were observed in terms of morphological, physiological and biochemical

analysis in the selected *Pongamia pinnata* species seedlings. It was found that the effect of salinity alone had great negative impact on biomass, leaves, chlorophyll, proline and protein content. The seedlings showed very good results under waterlogging treatment *i.e.* broad surface leaf area, highest photosynthetic rate and stomatal conductance, unaffected chlorophyll content and second highest proline and protein content which has impacted the highest biomass result. The presence of leaves for various analysis, highest proline and protein content, biomass and presence of two new protein bands across defensive proteins showed the tolerant behavior of seedlings under salinity + waterlogging better than salinity treatment alone. This clearly depicts the close relationship of species morphology with its physiological and biochemical condition and also shows the strong defensive response for protection and survival. Forest tree species are the most long-living organisms with the numerous tangible and intangible benefits. Hence, in order to protect the environment and livelihood and to fulfill the resource requirement of increasing population there is need of plantations of forest tree species with economically important attributes which should be suitable for such areas.

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