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Physiological and biochemical traits of adaptability in *Calophyllum inophyllum* (L.)

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Abstract
Calophyllum inophyllum L. is a potential tree borne oilseed gaining popularity for both medicinal and biofuel properties. Clonal variations with respect to physiological and biochemical traits in this species across three different locations such as Neyveli, Chennai and Salem was studied. The results indicated that *C. inophyllum* can with stand moderately harsh climatic conditions. It was evinced that to screen the adaptability of this species to dry climatic conditions, analysis of physiological traits such as Chlorophyll Stability Index (CSI) and Nembrane Injury Index (MII) and biochemical traits such as Chlorophyll contents, Proline and Phenols stand feasible. The present study indicated that clones C6 and C62 can withstand harsh climate compared to other clones studied. Clones C6 and C62 can therefore be deployed for vegetative multiplication for raising plantations with high productivity by farmers and forest departments.

Keywords: *Calophyllum inophyllum*, tree borne oilseed, adaptability, chlorophyll stability, membrane integrity, proline

Introduction
Low productivity of forest and considerable land use lead to the change from forest to agriculture in the past has resulted into enhanced biotic pressure on the existing forests. Therefore, it is crucial to increase the productivity of forest and also the area under tree cover to meet the growing demand for all kinds of timber, fuel, food, fodder, fibre within the country (Lal, 2007). Clonal technology is one such viable option to improve productivity especially in native tree species like *Calophyllu inophyllum*. *C. inophyllum* is a medium to large sized tree that belongs to Clusiaceae family. It is called as Alexandrian Laurel in English, and its vernacular names are Punnai (Tamil), Pouna (Telugu), Sultanachampa (Hindi), Punna (Malayalam) and Surahonne (Kannada). It is native to tropical Asia, east Africa and extends to Australia. It is widespread in countries like India, Malaysia, Indonesia, Srilanka, Phillipines, Myanmar, Taiwan, Thailand, Hawaii and other Paific Islands (Anandalakshmi, 2012) [2]. It grows well in sandy and well drained soils distributed upto an elevation of 800m and withstands temperature range of 18 to 33 ºC and rainfall of 1000 to 5000mm. In India, it is found along the sea coasts of the Indian Peninsula and the Andaman and Nicobar Islands. On the West Coast, it is found from Mumbai southwards to Southern Kerala and along the East Coast, from Orissa southwards (Anandalakshmi, 2014) [1].
C. inophyllum is known for its oil that possesses medicinal values and is also a potential biofuel (Sahoo *et al.,* 2009) [27]. Presently the Calophyllum oil, even without transesterification, has been proved very useful in running small motor engines, pumpsets etc. by farmers in Tamil Nadu. It is reported that the typical yield of an adult tree is around 5kg of cold pressed oil is produced for every 100kg of fruit (Friday and Okano, 2006) [13]. Its benefits and versatility are now increasingly known and accepted in the modern world. *C. inophyllum* is renowned for its remarkable healing properties and its oil has been used to treat diabetic sores, psoriasis, herpes and hemorrhoids (Dweck and Meadows, 2002) [12]. The seed kernel oil finds wide applications, such as luminant, lubricant, soap making apart from use as a medicated oil. The timber is used for beams, furniture, railway carriages and ship building (Shetty *et al.,* 2002) [29]. Realising the value of the multipurpose tree, at global level South

Pacific Regional Initiative on Forest Genetic Resources (SPRIG) has identified *C. inophyllum* as a priority species for genetic improvement in South East Asian countries (Pouru, 2000). In India, studies on distribution, genetic variation, selection and germplasm bank establishment can be taken up to initiate genetic improvement of the species (Krishnakumar *et al.*, 2010) [20].

At the Institute of Forest Genetics and Tree Breeding, Coimbatore, high fruit and oil yielding trees of *C. inophyllum* were identified, multiplied and established multilocation clonal trials at three places in Tamil Nadu, namely, Neyveli, Salem and Chennai inorder to assess the adaptability of these clones to different sites. The variability expressed in the physiological and biochemical traits which could serve as indicators for adaptive characters to tolerate dry conditions were analysed so that superior clones of *C. inophyllum* could be screened.

Materials and Methods

Collection of leaf sample

The fresh green leaves from the trees of five *C. inophyllum* clones – C6, C30, C62, C86, C88 were collected from the six year old multi location trials laid at Neyveli, Salem and Chennai. The details of the trials are as follows,

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Trial location	Altitude (msl)	Latitude	Longitude	Mean annual Rainfall (mm)	Maximum temperature °C	Soil type	Topography
Neyveli	87	11.61°N	79.44°E	1142	40	Sandy loam	Level ground
Chennai	28	12.79°N	80.04°E	1400	41	Sandy loam	Level ground
Salem	278	11.76°N	78.16°E	600	39	Sandy loam	Level ground

The leaves were surface cleaned and packed in polythene covers immediately after collection and used for the study.

Physiological traits

a. Relative Water content (RWC)

Fully expanded leaf samples were taken from the middle of the plant and used 5-6cm portion for recording Relative Water Content. RWC is calculated by the formula given by Barrs and Weathery (1962) and expressed in percentage.

$$RWC = \frac{\text{Fresh weight-Dry weight}}{\text{Turgid weight-Dry weight}} \times 100$$

b. Chlorophyll Stability Index (CSI)

CSI was estimated by following procedure of Koleyoreas (1958) and expressed in percentage.

$$CSI = \frac{\text{Total chlorophyll content (treated)}}{\text{Total chlorophyll content (Control)}} \times 100$$

c. Membrane Injury Index (MII)

Membrane Injury Index of leaves was calculated by the method of Blum and Ebercon (1981) [7]

$$MII = 1 - \frac{[EC \text{ at } 40\ ^\circ C]}{[EC \text{ at } 100\ ^\circ C]} \times 100$$

Biochemical Parameters

a. Estimation of Chlorophyll (Arnon *et al*., 1949) [3]

Chlorophyll is extracted in 80% acetone and the absorption at 663nm and 645nm are read in a spectrophotometer. Using the absorption coefficients, the amount of chlorophyll was calculated.

Mg chlorophyll a/g tissue = $12.7(A_{663}) - 2.69(A_{645}) \times V/1000 \times W$

Mg chlorophyll b/g tissue = $22.9(A_{645}) - 4.68(A_{663}) \times V/1000 \times W$

Mg total chlorophyll/g tissue = $20.2(A_{645}) + 8.02\ (A_{663}) \times V/1000 \times W$

Where

A = absorbance at specific wavelengths
V = final volume of chlorophyll extract in 80% acetone
W= fresh weight of tissue extracted

b. Estimation of Total Phenols (Malik and Singh, 1980) [24]

Phenols react with phosphomolybdic acid in folin-ciocalteau reagent in alkaline medium and produce blue coloured complex. The absorbance is noted at 650 nm. Standard curve using different concentrations of catechol was prepared, from which the amount of phenols was calculated.

c. Estimation of Proline (Bates *et al.*, 1973) [6]

During selective extraction with aqueous sulphosalicylic acid, proteins are precipitated as a complex. Other interfering materials are also presumably removed by absorption to the protein-sulphosalicylic acid complex. The extracted proline is made to react with ninhydrin in acidic conditions to form the chromophore (red colour) and read at 520nm.

d. Estimation of Ascorbic Acid (Harris and Ray, 1935) [14]

Ascorbic acid reduces the 2, 6-dichlorophenol indophenol dye to a colourless leuco-base. The ascorbic acid gets oxidised to dehydroascorbic acid. Though the dye is a blue coloured compound, the end point is the appearance of pink colour. The amount of the dye consumed is equivalent to the amount of ascorbic acid.

e. Estimation of Malondialdehyde (Dhinsa, *et al.*, 1981) [11]

The level of lipid peroxidation was measured following the thiobarbituric acid (TBA) test which determines malondialdehyde (MDA) as an end product of lipid peroxidation. Freshly harvested seedling sample (0.5g) was homogenized in 4 ml of 1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was added to 1.0 ml of 0.5% (w:v) thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 95^0C for 30 min and then quickly cooled in an ice bath. Again centrifuge it for another 10,000 rpm for 10 min, and read the absorbance at 532nm using a UV-VIS spectrophotometer. The value for non-specific absorption at 600 nm was subtracted from the value recorded at 532 nm. The MDA-TBA complex (pink pigment) content was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol $(MDA)g^{-1}$ fresh weight.

Statistical analysis

The experiments were conducted in Completely Randomized Design. The effect of insect control treatments and effect of

location (seed source) were analysed by two way ANOVA at 5% level of significance using GENSTAT 5.0 software. Prior to analysis the percentage data were transformed to arc sine values.

Results and Discussion

Variability in physiological traits of the clones of *Calophyllum inophyllum*

The three physiological traits studied for variations among the clones of *C. inophyllum* were Relative water content (RWC), Membrane injury index (MII) and Chlorophyll stability index (CSI). Relative water content (RWC) did not vary significantly across clones, locations and clones x locations. However, membrane injury index (MII) varied significantly across clones, locations and clones x location. Chlorophyll stability index (CSI) did not vary across locations alone. The CSI was found very high for C6 and C62 recording 1.078 and 1.169 respectively. MII was very high for C6 and C88 recording 37.94% and 41.21% respectively, and the lowest for C62 showing 29.3%. The results indicate that C62 is the superior clone based on CSI and MII. Across locations, clones established at Salem recorded the highest RWC of 74.07% and the highest MII of 39.22% (Tables 1a, 1b & Figures 1, 2).

Table 1a: ANOVA for physiological traits of *C. inophyllum* clones

Physiological Parameter	Source of variation	Sum of squares	Mean square	F probability	Significance
Relative Water Content (RWC)	Clones	82.27	20.57	0.606	NS
	Location	149.86	74.93	0.094	NS
	Clones*Location	185.02	23.13	0.630	NS
Chlorophyll Stability Index (CSI)	Clones	7689.8	1922.5	<.001	S
	Location	1.6	0.8	0.996	NS
	Clones*Location	13474.2	1684.3	<.001	S
Membrane Injury Index (MII)	Clones	909.29	227.32	<.001	S
	Location	695.35	347.68	<.001	S
	Clones*Location	1361.98	170.25	<.001	S

Table 1b: Mean values for physiological traits of *C. inophyllum* clones

Physiological Parameter	Clones	Relative Water Content	Chlorophyll Stability Index	Membrane Injury Index
Neyveli	C6	67.26	1.25	34.46
	C30	70.82	1.14	45.23
	C62	71.45	1.23	26.00
	C86	74.54	1.21	38.42
	C88	73.20	0.57	45.72
Chennai	C6	71.94	0.63	41.39
	C30	68.36	0.59	30.44
	C62	71.69	0.63	23.22
	C86	69.13	1.71	32.90
	C88	70.30	1.30	29.31
Salem	C6	72.93	1.47	37.99
	C30	73.34	1.04	33.60
	C62	76.10	0.75	38.68
	C86	70.78	0.73	37.26
	C88	77.19	0.61	48.58
Grand mean		71.94	1.00	36.21
Mean values for Clones				
C6		70.71^{a}	1.078^{a}	37.94^{a}
C30		70.84^{a}	0.856^{c}	36.42^{b}
C62		73.08^{a}	1.169^{a}	29.30^{c}
C86		71.48^{a}	0.907^{b}	36.19^{b}
C88		73.56^{a}	0.990^{b}	41.21^{a}
Mean values for Location				
Neyveli		71.46^{b}	0.999^{a}	37.96^{a}
Chennai		70.29^{b}	0.999^{a}	31.45^{b}
Salem		74.07^{a}	1.002^{a}	39.22^{a}
Standard error of differences (s.e.d.)	Clones	2.237	0.057	1.962
	Location	1.732	0.044	1.520
	Location*Clones	3.874	0.099	3.398
Least significant differences (l.s.d.)	Clones	4.505	0.115	3.952
	Location	3.489	0.089	3.061
	Location*Clones	7.802	0.199	6.844

NS – Not Significant; S – Significant; Means with the same letter do not differ significantly at p<0.05%

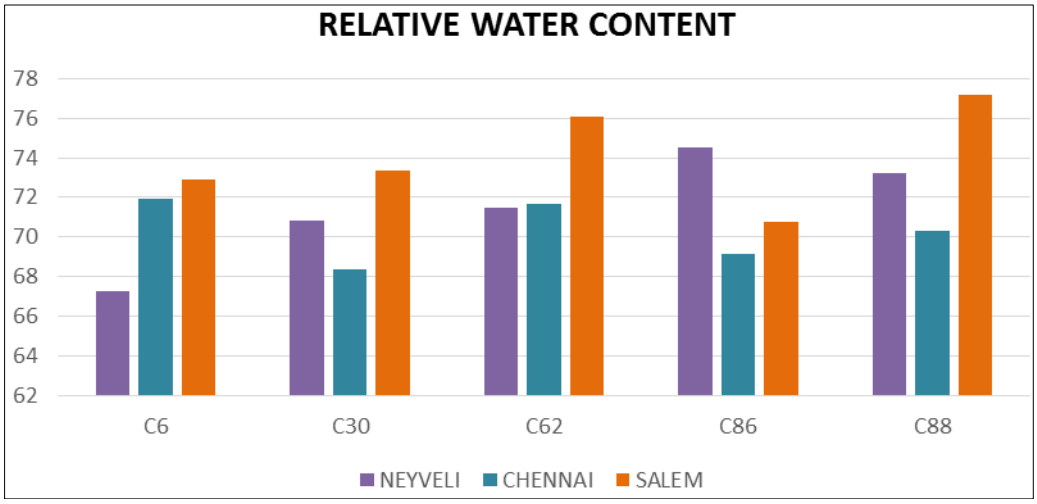

Fig 1: Relative Water Content (%) in leaves of C. inophyllum clones across locations

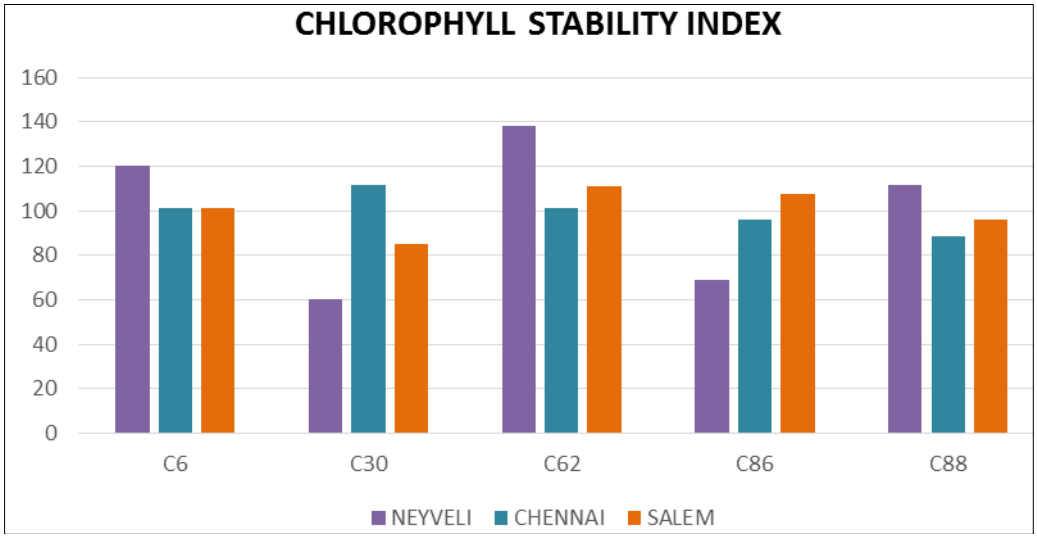

Fig 2: Chlorophyll Stability Index in leaves of C. inophyllum clones across locations

From the above study it is evident that Chlorophyll Stability Index (CSI) and Membrane Injury Index (MII) stand as dependable traits to evaluate the adaptive capacity of *C. inophyllum* clones. Chlorophyll stability is a function of temperature and it is found to correlate with drought tolerance. Chlorophyll stability index is a measure of integrity of membrane or heat stability of the pigments under stress conditions (Kaloyereas, 1958). The CSI is a single parameter used to measure frost or drought resistance of a plant. Sairam *et al*. (1996) [28] reported that both drought stress and temperature stress decreased membrane stability, chlorophyll content and chlorophyll stability index in all wheat genotypes. The high chlorophyll stability indices help the plants to withstand stress through better availability of chlorophyll. This leads to increased photosynthetic rate and more dry matter production (Madhan Mohan *et al*., 2000) [23]. Relative water content of leaf (RWC) indicates the actual water content to its maximum turgidity. It was observed that, plants under zero stressed condition had maintained higher RWC throughout the course than strong stressed and severe stressed plants (Kardile, 2018) [18]. Upreti *et al*., (1997) [31] noted changes in RWC under stress and normal conditions, the reduction being significant under stress condition. The higher CSI, higher RWC and lower MII values in the present study reveals that this species is able to sustain under stressed conditions like drought and high temperatures.

Variability in biochemical traits of the clones of Calophyllum inophyllum

Among the various biochemical parameters, phenols, ascorbic acid and malondialdehyde (MDA) contents did not vary significantly across the clones, whereas chlorophyll contents and proline varied for the clones. Chlorophyll b alone did not vary significantly across the locations. The interactive effect of clones and locations were found varying for all the biochemical parameters studied except for ascorbic acid and MDA (Tables 2a, 2b & Figures 3-8).

In this study, based on the interactive effects of clones x locations Chlorophylls A and B, Total Chorophyll, Phenols and Proline could be used to screen adaptive clones. Most of these biochemical traits were found high for clones C62 and C6 which could be ranked superior for adaptability across locations among the five clones taken up for the study.

Table 2a: ANOVA for biochemical traits of C. inophyllum clones

Biochemical parameter	Source of variation	Sum of squares	Mean square	F probability	Significance
Chlorophyll a	Clones	0.68459	0.17115	<.001	S
	Location	1.69156	0.84578	<.001	S
	Clones*Location	1.11899	0.13987	<.001	S
Chlorophyll b	Clones	0.13576	0.03394	0.007	S
	Location	0.03796	0.01898	0.114	NS
	Clones*Location	0.28875	0.03609	<.001	S
Total chlorophyll	Clones	1.25851	0.31463	<.001	S
	Location	2.09396	1.04698	<.001	S
	Clones*Location	2.30352	0.28794	<.001	S
Phenols	Clones	155.18	38.79	0.052	NS
	Location	211.12	105.56	0.002	S
	Clones*Location	262.42	32.80	0.049	S
Proline	Clones	0.00281	0.00070	0.002	S
	Location	0.02672	0.01336	<.001	S
	Clones*Location	0.00742	0.00093	<.001	S
Ascorbic acid	Clones	4570.4	1142.6	0.193	NS
	Location	22161.2	11080.6	<.001	S
	Clones*Location	9927.6	1241.0	0.118	NS
Malondialdehyde	Clones	43.66	10.91	0.780	NS
	Location	221.63	110.82	0.017	S
	Clones*Location	324.96	40.62	0.142	NS

NS – Not Significant S - Significant

Table 2b: Mean values for biochemical traits of C. inophyllum clones

Biochemical parameters	Clones	CHL. A mg/g	CHL. B mg/g	Tot. CHL mg/g	Phenols mg/g	Proline mg/g	Asc. acid mg/100g	MDA nmol/g
Neyveli	C6	1.10	0.52	1.62	18.14	0.083	58.33	11.05
	C30	0.48	0.32	0.81	19.64	0.076	53.33	15.75
	C62	1.19	0.66	1.85	14.03	0.088	75.00	13.13
	C86	0.49	0.43	0.92	18.45	0.055	61.67	11.65
	C88	0.94	0.56	1.50	13.30	0.059	61.67	11.44
Chennai	C6	0.53	0.52	1.04	11.57	0.057	56.67	18.02
	C30	0.55	0.60	1.15	9.32	0.027	53.33	12.06
	C62	0.52	0.53	1.04	10.25	0.016	36.67	16.09
	C86	0.49	0.50	0.99	14.32	0.055	35.00	18.85
	C88	0.49	0.42	0.91	16.88	0.020	56.67	16.84
Salem	C6	0.48	0.50	0.90	14.10	0.019	123.33	13.00
	C30	0.41	0.36	0.76	13.38	0.018	100.00	12.52
	C62	0.47	0.52	0.99	8.80	0.032	50.00	9.27
	C86	0.45	0.51	0.96	12.31	0.024	93.33	8.25
	C88	0.47	0.38	0.86	16.74	0.018	101.67	17.17
Grand mean		0.60	0.49	1.09	14.08	0.043	67.78	13.67
Mean values for Clones								
C6		0.702^{a}	0.512^{a}	1.187^{a}	14.60^{a}	0.0528^{a}	79.4^{a}	14.02^{a}
C30		0.479^{b}	0.432^{b}	0.903^{c}	14.12^{a}	0.0403^{c}	68.9^{a}	13.44^{a}
C62		0.725^{a}	0.569^{a}	1.296^{a}	11.03^{b}	0.0456^{b}	53.9^{b}	12.83^{a}
C86		0.477^{b}	0.479^{b}	0.955^{b}	15.03^{a}	0.0446^{b}	63.3^{a}	12.92^{a}
C88		0.634^{a}	0.458^{b}	1.090^{b}	15.64^{a}	0.0320^{d}	73.3^{a}	15.15^{a}
Mean values for Location								
Neyveli		0.839^{a}	0.501^{a}	1.338^{a}	16.71^{a}	0.0719^{a}	62.0^{b}	12.60^{b}
Chennai		0.156^{c}	0.514^{a}	1.028^{b}	12.47^{b}	0.0350^{b}	47.7^{b}	16.37^{a}
Salem		0.457^{b}	0.455^{c}	0.892^{c}	13.06^{b}	0.0221^{c}	93.7^{a}	12.04^{b}
Standard error of differences (s.e.d.)	Clones	0.0473	0.0372	0.0692	1.591	0.0049	10.94	2.035
	Location	0.0366	0.0288	0.0536	1.233	0.0038	8.48	1.576
	Location*Clone	0.0819	0.0645	0.1199	2.756	0.0084	18.95	3.525
Least significant differences (l.s.d.)	Clones	0.0952	0.0750	0.1394	3.205	0.0098	22.04	4.099
	Location	0.0738	0.0581	0.1080	2.482	0.0076	17.07	3.175
	Location*Clone	0.1649	0.1298	0.2414	5.551	0.0169	38.18	7.099

Means with the same letter do not differ significantly at p<0.05%

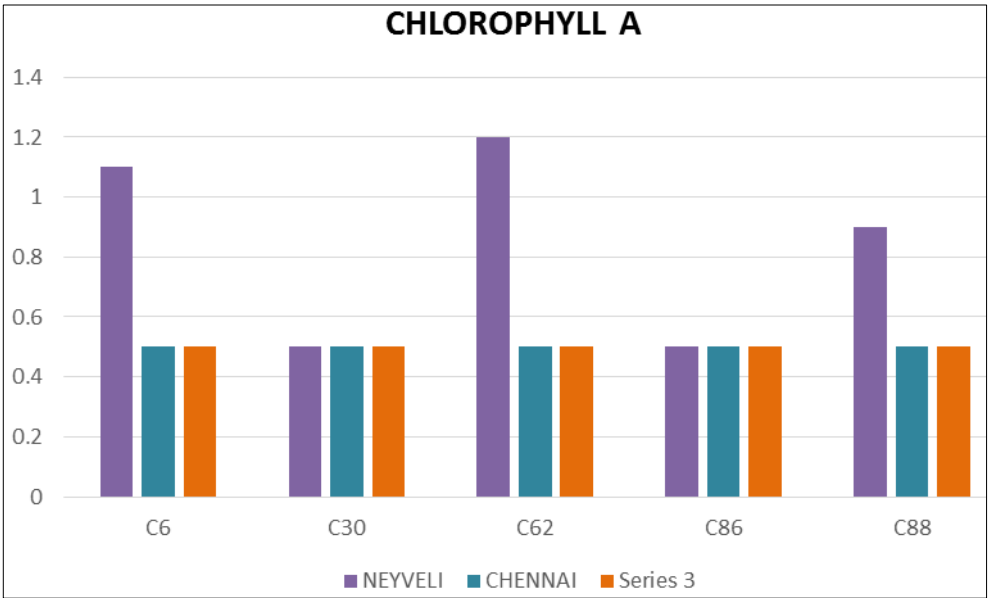

Fig 3: Chlorophyll A in leaves of C. inophyllum clones across locations

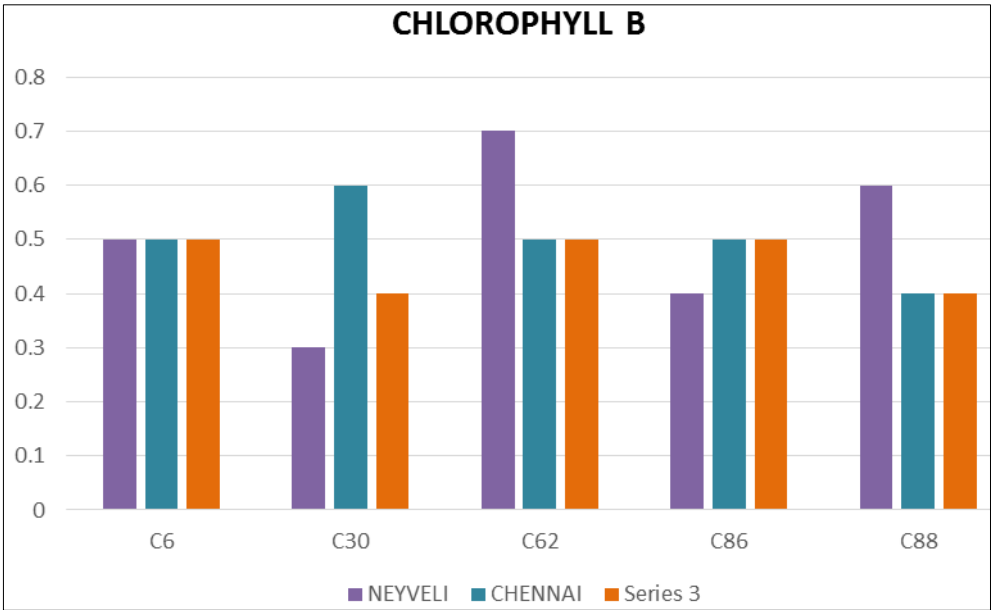

Fig 4: Chlorophyll B in leaves of C. inophyllum clones across locations

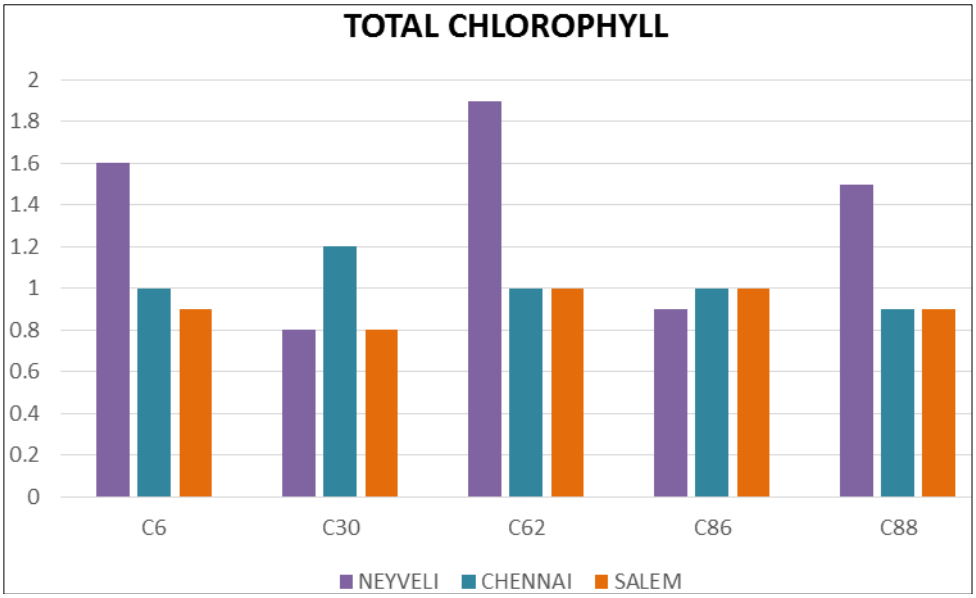

Fig 5: Total Chlorophyll in leaves of C. inophyllum clones across locations

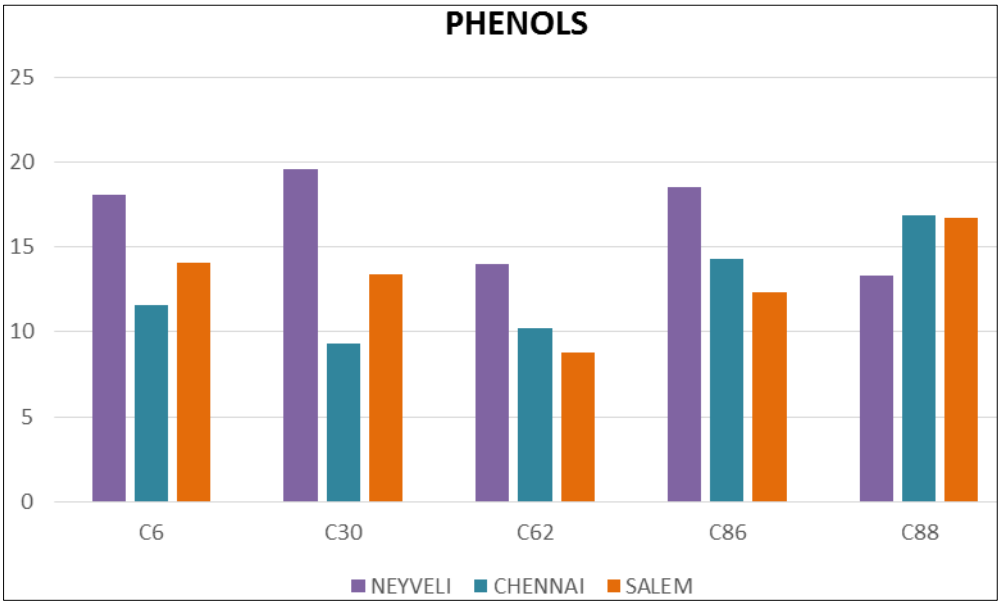

Fig 6: Phenols in leaves of C. inophyllum clones across locations

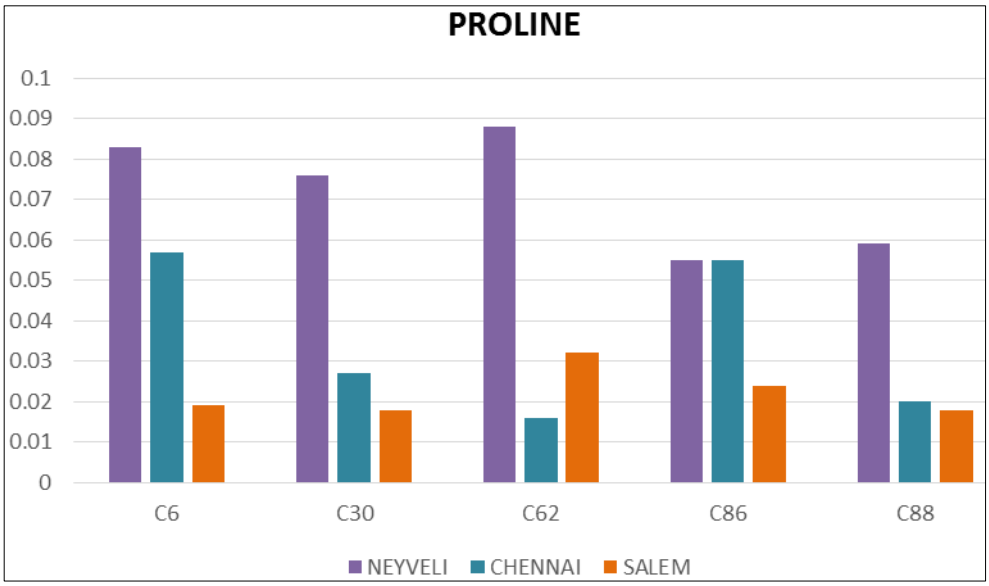

Fig 7: Proline in leaves of C. inophyllum clones across locations

In *C. inophyllum* Hathurusingha *et al*., (2011) [15] has reported variations in oil content across various seed sources. However studies on clonal variations in biochemical traits is very scanty. Chlorophyll is an essential pigment for photosynthesis, utilizing the energy of photons for redox reactions (Baker, 2008) [4]. Thus, leaf chlorophyll concentration (Chl) may directly influence the photosynthetic capacity of plants (Croft *et al*., 2017) [9]. From the table 3a and 3b it was evident that highest chlorophyll contents were recorded for C62. Similarly, across locations, Neyveli recorded the better variation compared to other two locations. From the result it can be suggested that C6 and C62 show good performance.

Phenolic compounds play important roles in plant growth and development, particularly in defense mechanisms. Most of the phenolic compounds have potent antioxidant properties, neutralizing the effects of oxidative stress. Some of them exhibit ability to chelate heavy metal ions (Kamila Kulbat, 2016). C62 contained comparatively less phenolic compounds across the clones and Neyveli recorded high phenols across locations.

Water deficit stress often causes an increase in proline accumulation in plant leaves (Dashek and Ericson, 1981) [10]. The enhancement level of proline is believed to be of adaptative significance. Resistance to water deficit stress occurs when plants withstand the imposed stress, and may arise from either tolerance or avoidance of dehydration (Levitt, 1980) [22]. Proline is a non-toxic compatible osmolyte which may alleviate the deleterious effects of stress on enzyme activity and the structure of cell membranes. It has been indicated that proline lowers the generation of highly destructive free radicals species (Smirnoff and Cumbes, 1989) [30]. C6 recorded high proline content across clones and similarly, Neyveli location recorded contained high quantities of proline.

L-Ascorbic acid is a highly abundant metabolite and has important roles in plant stress physiology as well as growth and development. In the detoxification of reactive oxygen species, it is a key antioxidant. As an enzyme cofactor, it

plays significant parts in photoprotection, the wounding response, and insect herbivory as well as cell expansion and division (Conklin, 2001) [1]. The *C. inophyllum* clones did not show any significant variation in ascorbic acid content. However, Salem has recorded higher content of ascorbic acid across locations.

Lipid peroxides are disintegrated quickly and form reactive carbon compounds. MDA is an important reactive carbon compound which is used commonly as an indicator of lipid peroxidation (Jacob and Burri, 1996) [16]. MDA in the samples is important for the evaluation of oxidative stress in biological systems (Nordberg and Arner, 2001) [25]. Though Chennai showed high quantities of MDA across the locations, there was no significant variation in MDA contents across the clones.

It could also be observed that Neyveli, Chennai and Salem record high temperatures upto 40 °C during summers and the mean rainfall is also not very luxuriant making the environment in these locations moderately harsh and dry. Despite these conditions some clones of *C. inophyllum* could establish and perform well indicating that this species can adapt to moderately harsh climatic conditions and can be domesticated.

Conclusion

From the study it could be evinced that screening *Calophyllum inophyllum* clones for traits such as Chlorophyll Stability Index (CSI), Membrane Injury Index (MII), biochemical traits such as Chlorophyll, Proline and Phenols could help us screen climate hardy clones that can adapt well to harsh climatic conditions. In general, based on the physiological and biochemical variability studies, C6 and C62 are superior compared to other clones and their performance across locations are fairly good. Hence, these two clones can be recommended for vegetative propagation on large scale and used for establishing plantations by State Forest Departments and farmers in agroforestry mode for better productivity.

Fig 8: Calophyllum inophyllum trial plot at Neyveli

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