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Research Note

Prospective functional indicators of carbon sequestration potential in *Casuarina equisetifolia* L.

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Abstract
Chlorophyll contents reflects the plants ability to adapt to stress, and has been used as a diagnostic tool to study genotypic variation. Fifteen clones of *Casuarina equisetifolia* L. selected from varied climatic conditions were compared for their growth performance and biochemical profile in order to identify markers for early screening of perennials for their adaptation to changing climatic conditions. Chlorophylls a and b, total chlorophyll, a:b ratio and carbonic anhydrase were chosen as indicators of biochemical alteration. The main questions addressed in this study were (1) Do clones show variations at the biochemical level, and can it be correlated with growth performance? (2) Which biochemical indicator is more effective as a marker for productivity and consequently in predicting carbon accumulation in clones? Significant variations were observed in all the parameters studied. The chlorophyll a and the hydrolytic enzyme, carbonic anhydrase (CA) activity correlated positively with DBH, a measure of the carbon accumulation in plants suggesting their application as a sound method to test seedling stock quality for enhanced sequestration potential.

Keywords
Functional indicators; Chlorophyll; Carbonic anhydrase; carbon accumulation; *Casuarina equisetifolia*

Global warming is a problem of great concern. Large scale plantation of trees (carbon sequestration) is one of the means of removing excess atmospheric carbon-dioxide. Planting new forests, rehabilitating degraded forests and enriching existing forests contribute to mitigating climate change as these actions increase the rate and quantity of carbon sequestration in biomass (Karsenty et al., 2003). Casuarina equisetifolia (Family: Casuarinaceae) was introduced into peninsular India in the 19th century to fuel steam locomotives (Midgley et al., 1983) and is now extensively planted for wood production, shelterbelts and land rehabilitation (Kondas, 1983). The timber finds use as pole for construction of scaffoldings. It is also an excellent fuelwood. Of late the wood is also used by the paper and pulp industries. C. equisetifolia is an excellent wind break and can considerably reduce wind damage and increase crop yield. It is the most preferred species for raising coastal shelterbelts. The main advantage of planting casuarinas is the fixation of atmospheric nitrogen due to its association with Frankia, in the root nodules, thus enriching the soil with nitrogen. Marcolin et al. (2002) have shown that Casuarinas are very efficient at carbon sequestration due to their fast growth rate and adaptability.

Tree improvement in Casuarina equisetifolia is on since 1980s in various parts of India. Large number of clones have been identified for their productivity and used in the establishment of clonal seed orchards. If the clonal variation in productivity and in turn carbon sequestration potential is assessed, the most productive clones could be used for raising clonal plantations as well as clonal seed orchards, aimed at higher productivity and thus greater carbon sequestration.

Plants react to changes in light conditions in terms of quantity and composition of chloroplastidic pigments, which affects the photosynthetic properties and consequently the accumulation of plant biomass (Morais et al., 2007). Chlorophyll a fluorescence helps to study the conversion and transfer of energy in photosystem II (Gonçalves et al., 2001; Gonçalves and Santos Junior, 2005; Ribeiro et al., 2004). Chlorophyll fluorescence has, thus, been deployed as a useful physiological test to detect perturbation of leaf metabolism and growth of seedling to select high-quality seedlings or clones for a particular environment (Barbagallo et al. 2003). Carbonic anhydrase (CA), or Carbonate dehydratase EC 4.2.1.1, is the only enzyme of photosynthesis involved in carbon capture. In chloroplast of plant cell, its role is related to photosynthetic fixation of carbon-dioxide (Lindskog and Coleman, 1973). Any change in CA activity directly affects the rate of photosynthesis and thus carbon-dioxide fixation. CA in different plant species has been studies by Waygood and Clendenning (1950) who found that physiological changes occurring in leaves

preceding autumnal colouration would affect carbonic anhydrase activity.

The present study was an attempt to correlate growth, photosynthesis regulation and CA activity in clones of Casuarina equisetifolia selected for productivity to explore whether, CA, the enzyme associated with carbon-dioxide capture, can serve as a biochemical indicator for carbon sequestration potential, for early screening to avoid elaborate field testing.

Casuarina equistifolia selected from various parts of India have been assembled as clones in a clone bank at the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore (Latitude11^{o} 01' N, Longitude 77^{o} 02' E). Fifteen clones identified as suited to problem soils (coastal areas and mine dumps in Tamil Nadu at a selection intensity of 1 in 10,000) based on survival and productivity (Jayaraj et al., 2001) were selected for the study.

Biochemical analysis

A random of five ramets were sampled from each clone. Sample extraction was carried out with different types of solvents for various analyses (phosphate buffer and 80% acetone) centrifuged and then supernatant was taken for the estimation. Chlorophylls were extracted into solution with 80 per cent acetone, and absorbance measured at 645, 652 and 663 nm to determine the total chlorophyll, chlorophylls a and b contents (Yoshida et al., 1976). Chlorophyll a:b ratio was also calculated. Carbonic anhydrase activity was measured following Warrier et al. (2014).

Growth measurements:A trial was laid out comprising these clones at Model Nursery, IFGTB, Coimbatore to assess the performance of the clones. At the end of twenty months, total height (H) (m), diameter at breast height (DBH) measured at 1.37m from the ground level using an electronic digital vernier calliper and expressed in centimeter and Volume index (D^2H) used as a surrogate for total tree volume (Elliott et al., 2002; Luna and Singh, 2007) expressed in m^3 were measured.

Statistical analysis:All experiments had a complete randomized design. Data obtained was subjected to Analysis of variance (ANOVA) and means separation done by Duncan's Multiple Range mean separation test (DMRT) using the SPSS Ver.10.0 package wherever significant. Cluster analysis based on the different biochemical parameters in relation to height, diameter and volume was done to find linear combinations to discriminate between the groups.

There were significant variations in all the parameters among the fifteen clones studied (Table 2). Five groups emerged when clones were grouped based on chlorophyll a content. Clones TNPP3 and TNPB1 recorded highest chlorophyll 'a' activity while the least was recorded in TNKP3. Total chlorophyll was highest in TNRM4, TNRM7, TNPP2 and TNPB1. Here again lowest levels were recorded in TNKP3. Based on total chlorophyll content the clones segregated into five groups. TNMT6 recorded the highest levels of chlorophyll b activity while the least was recorded in TNRM1. Based on chlorophyll b content three groups were formed. The ratio of chlorophyll a to chlorophyll b should be ideally in the ratio of 3:1. In Casuarina equisetifolia clones it was found to be in the ratio of 2:1 (Based on mean values). Based on this ratio they were grouped into five groups. The ratio of chlorophyll a to b was highest in TNRM1 while clones TNMT6 and TNRM7 recorded low values.

Work on chloroplast fragmentation reveals that chlorophyll a and chlorophyll b are associated with Photosystems I and II in photosynthesis. Chlorophyll a:b ratio of 2 is primarily responsible for system II, and a chlorophyll a: b ratio of 6 is responsible for system I (Boardman and Anderson, 1964; Fork et. al., 1964). The normal ratio of chlorophyll a to b in the chloroplast (3:1) is observed to vary with changes in light absorption, stress or when there is a shift in photosystems during the process of photosynthesis. In the present study, only clone TNRM1 showed a near normal a:b ratio while most of the clones had values nearing two suggesting an active system II. Clone TNCN1 had low a:b ratio, coupled with low CA activity and its overall growth performance was also poor. Clone TNMT6 which had the lowest a: b ratio (1.37), however, had high CA activity and its growth performance was also good. Hence a:b ratio and chlorophyll a levels are reliable indicators of growth potential which in turn reflects productivity. Clones with high chlorophyll a and b contents with reduced a: b ratio confirm the shift in photosystem and hence the reason for high productivity.

Duncan's multiple range test (DMRT) grouped the fifteen clones of Casuarina equisetifolia based on CA activity into 9 groups. Lowest CA activity was observed in TNKP2 and TNCN1 while the clones TNMT6, TNPB1 and TNRM5 recorded high activity.

Carbonic anhydrase (CA; EC 4.2.1.1) catalyses the interconversion of CO_2 and HCO_3^- and is a major protein constituent of the C3 higher plant chloroplast where it is presumed to play a role in photosynthetic carbon assimilation. In leaves, CA represents 1–20 % of total soluble protein with abundance next only to ribulose-1,5-bisphosphate

carboxylase (Rubisco) in chloroplast. It facilitates CO_2 supply to PEP carboxylase in C4 and CAM plants and to Rubisco in C3 plants, rather than CO_2 as the inorganic carbon substrate. To sustain this process, atmospheric CO_2 entering mesophyll cells must be rapidly converted to HCO_3^- and this reaction should rightly be regarded as the first step in C3 and C4 photosynthesis (O'Leary, 1982). The fifteen clones showed significant variations in the CA activity suggesting variation in the species in terms of production of this hydrolytic enzyme. This enzyme being the only one in the whole of the photosynthesis cascade to be involved in the transport of CO_2, assumes significance as the clones could be categorized into clusters based on the activity of this crucial enzyme.Of the growth parameters recorded, total height ranged from 8.03 to 10.83 m with a mean of 9.66 ± 0.80 m (mean ± standard deviation). TNRM2 recorded the maximum height (10.83 m) while TNKP3 recorded the minimum height (8.03 m). Diameter at breast height was highest for TNRM-7 while the least was recorded in TNCN-1. The values ranged from 4.7 to 8.0 with a mean of 6.18 cm and SD 0.8. The range of volume index was between 198 and 691 with a mean and SD of 404.47 ± 148.09 (Table 3).

Correlation analysis, an indication of the degree of closeness of the relationship between two variables (Table 4) showed no correlation between CA and chlorophyll contents. However, DBH significantly correlated with CA activity and chlorophyll a levels (0.540 and 0.541 respectively). Interestingly, no significant correlation was observed between CA and chlorophyll values in the present study. Despite this, the grouping patterns of the clones in relation to Chlorophyll and CA activity were similar. This might be attributed to the distribution of the enzyme both within and outside the chloroplasts for formation of CO_2 from bicarbonate ions at the chloroplast surface and a further reverse reaction within the chloroplast to obtain maximum rates of photosynthesis (Everson, 1970). It was observed that clones which performed well with respect to CA activity also exhibited higher chlorophyll content and good growth.

Correlation between a, b, total and a:b ratio was significantly high. Similarly, height DBH and volume index were correlated. Being dependent / derived parameters it was but expected that these parameters would be significantly correlated. However, to identify a functional indicator, it is essential to be able to correlate two entirely independent traits through a common linking factor. In this case, it was observed that DBH, a major factor in tree species for productivity estimations showed strong positive correlations with CA and chlorophyll. Valladares et al., (2000) describe how productivity in higher plants is controlled by leaves and elaborates on the involvement of leaf traits in adaptations of plants to the environment. An assessment of the quantity and composition of pigments in the chloroplasts reflects the plants response to changing light conditions. Carvalho et al., (2006) state that higher chlorophyll a contents and low a: b ratio is an indication of either low light intensities or the adaptation of a species to stabilize the light absorbance between photosystem I and II. Contrarily, when there is high irradiance of light, the a:b ratio increases due to the inter-relationship between chlorophyll a and b (Walters, 2005). Thus assessment of chlorophyll a fluorescence enables understanding the conversion, and the transfer of energy in photosystem II (Ribeiro et al., 2004) of a species. Further, changes in the ratios of Chl a/b can be considered as an adaptation to changing light conditions. An increasing ratio of Chl a/b at high irradiance favors the increase of PSII units and the efficiency of energy conversion (Walters, 2005). In the present study, the environmental conditions to which the different clones were subjected to remained the same. However, significant differences were observed in their a: b ratios with values ranging from 1.37 to 2.66. This suggests the varying sensitivity of clones to light intensity.

CA has an active role in photosynthesis, which is established by its presence in all photosynthesizing tissues. This in turn, increases the size of the source-to- sink allocation which produces more photo-assimilates and presumably results in a greater growth potential. Thus CA indirectly influences the growth of plants. Khan (1994) found a correlation between CA activity and photosynthetic rate and productivity in mustard, suggesting that CA could serve as a marker of productivity because the enzyme helps in carbon sequestration. Similar results have been observed in teak also (Tiwari et al., 2006) wherein CA has been used to study the photosynthetic ability of the species. The present study in Casuarinas corroborates the findings in teak that CA influences productivity. The CA activity and chlorophyll a contents were positively correlated with DBH, suggesting their potential application as an early screening marker for productivity in Casuarinas.

Clustering of clones based on D^2 analysis (Table 5) showed that the entire set of clones could be grouped into four clusters. Clone TNRM3 formed an isolated cluster. This clone had low CA activity though the chlorophyll activity exhibited was high. Similarly, it expressed lower DBH though height was comparable with other superior clones. A decrease in the ratio as in the case of TNMT 6 with increased chlorophyll b levels along with an increased CA activity and a complementing high

biomass suggests the involvement of these parameters in carbon sequestration. Further, these functional indicators can serve as a powerful tool in to study carbon sequestration and environmental amelioration in the current global warming scenario linked with elevated CO_2 concentrations.

Thus, CA and chlorophyll qualify as functional indicators of productivity in Casuarina which in turn could be translated as the carbon sequestration ability of the species. Selection of plants for high rate of growth and thus carbon sequestration in Casuarina equisetifolia can therefore be attempted at an early stage by measuring the CA and chlorophyll activity. The data indicate that the carbon sequestration potential among the selected fifteen clones is extremely varying and therefore scope exists for selection of clones for carbon sequestration potential and use in breeding programmes. Since the clones have been tested under uniform environmental conditions, the biochemical variations could be attributed to the genetic variation in the selected clones, the present work concludes that these ecophysiological differences among clones might be considered for the establishment of productive, sustainable and management of plantations. It also proposes application of these functional indicators as a early selection markers for carbon sequestration potential in Casuarina equisetifolia.

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Table 1. Details of *Casuarina equistifolia* clones used for the study

Code	Source	Location (Lat- Long)	Range of Rainfall/ year	Soil Type
TNRM1	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM2	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM3	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM4	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM5	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM7	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNRM8	Rameswaram	9°14'N-79°18'E	801-1000 mm	Sandy coast
TNPP 2	Pudupattinam	11°19'N-79°49'E	1001-1200 mm	Sandy coast
TNPP 3	Pudupattinam	11°19'N-79°49'E	1001-1200 mm	Sandy coast
TNKP2	Kelampakkam	12°47'N-80°13'E	1001-1200 mm	Red soil
TNCN1	Thaiyur	12°46'N-80°11'E	1001-1200 mm	Red soil
TNMT6	Maduranthakam	12°30'N-79°53'E	1001-1200 mm	Red soil
TNPB 1	Poovalambedu	13°20'N-80°08'E	1001-1200 mm	Red soil
TNAM1	Agaram Medu	13°16'N-80°11'E	1001-1200 mm	Red soil

Table 2: Chlorophyll and Carbonic Anhydrase activity in clones of *Casuarina equisetifolia*

Clone No.	Chlorophyll a		Chlorophyll b		Total Chlorophyll		Chlorophyll a :b		Carbonic Anhydrase	
	Mean	DMRT	Mean	DMRT	Mean	DMRT	Mean	DMRT	Mean	DMRT
TNRM1	1.80	de	0.69	c	2.45	e	2.66	a	1.56	fg
TNRM2	2.13	bc	0.93	abc	3.06	cd	2.32	abc	3.76	bc
TNRM3	2.14	bc	0.91	bc	3.05	cd	2.37	ab	1.13	gh
TNRM4	2.29	ab	1.33	abc	3.62	ab	1.76	bcde	1.91	f
TNRM5	2.30	ab	1.09	abc	3.39	bc	2.15	abcd	4.45	a
TNRM7	2.29	ab	1.54	ab	3.83	a	1.51	de	2.68	e
TNRM8	1.95	cd	0.98	abc	2.90	d	2.07	abcde	1.81	f
TNPP2	2.32	ab	1.52	ab	3.82	a	1.55	cde	2.83	de
TNPP3	2.36	ab	1.22	abc	3.57	ab	1.94	abcde	1.68	fg
TNKP2	1.93	cd	1.60	ab	2.88	d	1.60	bcde	0.52	i
TNKP3	1.54	e	0.80	bc	2.34	e	1.93	abcde	1.99	f
TNCN1	1.67	e	1.02	abc	2.69	de	1.65	bcde	0.61	hi
TNMT6	1.96	cd	1.72	a	3.07	cd	1.37	e	4.53	a
TNPB1	2.44	a	1.32	abc	3.76	ab	1.90	abcde	4.09	ab
TNAM1	2.11	bc	0.97	abc	3.08	cd	2.19	abcd	3.32	cd
Mean	2.08		1.18		3.17		1.93		2.46	
SE	0.08		0.23		0.12		0.22		0.18	

*Values sharing same superscripts do not significantly differ from each other

Table-3: Growth performance of the clones raised as a clonal trial

Code	Height (m)	DBH (cm)	Volume Index (cm^3)
TNRM1	9.85	6.43	456
TNRM2	10.83	7.12	577
TNRM3	10.52	6.13	432
TNRM4	9.90	6.68	464
TNRM5	10.31	7.86	678
TNRM7	10.42	8.00	681
TNRM8	10.13	5.73	344
TNPP2	9.59	6.19	382
TNPP3	8.67	5.10	228
TNKP2	10.12	5.65	348
TNKP3	8.03	5.03	205
TNCN1	8.61	4.70	196
TNMT6	10.04	6.24	401
TNPB1	8.98	6.52	409
TNAM1	8.91	5.27	266

Table-4: Correlation among the parameters studied

	CA	CHLA	CHLB	TOTAL	Height	DBH	Volume Index	A2B
CA	1.000							
CHLA	0.447	1.000						
CHLB	0.228	0.425	1.000					
TOTAL	0.403	0.933**	0.613*	1.000				
Height	0.183	0.318	0.198	0.209	1.000			
DBH	0.540*	0.541*	0.233	0.500	0.725**	1.000		
Volume Index	0.473	0.487	0.175	0.424	0.792**	0.989**	1.000	
A2B	-0.053	-0.082	-.857**	-0.390	0.141	0.056	0.117	1.000

* Significant at the 0.05 level (2-tailed).** Significant at the 0.01 level (2-tailed).

Table 5. Cluster members grouped based on D^2 analysis

Clusters	Means	Clones
Cluster I	6.33	TNRM1, TNRM2, TNPP3, TNKP2, TNAM1
Cluster II	4.87	TNRM4, TNRM5, TNRM7
Cluster III	11.38	TNRM3
Cluster IV	9.06	TNRM8, TNPP2, TNKP3, TNCN1, TNMT6, TNPB1