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Effect of availability of nutrition on determining the representation of sexual behaviour by *Cedrus deodara* (Roxb.) G. Don

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Abstract
In recent years, trends in plasticity of sex expression in conifers, particularly in gymnosperms has become a very intriguing subject for research. However, due to the limited research on this subject, the mechanisms underlying unpredictable gender expression remain unknown. *Cedrus deodara*, predominantly characterized as monoecious and occasionally dioecious, exhibits irregular intervals in cone production, with certain populations yielding seeds after three to four years. This unpredictable reproductive behavior is influenced not only by climatic factors but also by physiological factors, the specifics of which are yet to be elucidated. The present study aims to unravel the role of biochemical factors affecting periodic cone production in *Cedrus deodara*. Three distinct sites namely, Cheog forest in Theog, and HFRI Campus forest in Shimla, Himachal Pradesh and Chakrata range, Uttarakhand, each representing a pure stand of the species were selected and 300 trees at each site were marked for recording reproductive behaviour of the species. Selection criteria were based on the reproductive age of *Cedrus deodara*, as it attains maturity when it reaches a height of 19 to 20 meters. Our findings revealed that *Cedrus deodara* exhibits subdioecious behaviour, characterized by the occurrence of four basic sex forms such as male trees, female trees, monoecious trees, and neutral trees. Additionally it was also found that individual trees demonstrated change in their expression of sex during each reproductive cycle. Samples from trees showing uncertainity in their expression of gender were collected for exploring the effect of total carbohydrate content and total protein content on this pattern. We found that the allocation of energy resources like carbohydrates and proteins were directly proportional to the production of seeds, the previous year.

Keywords: Conifers, carbohydrate content, protein content, cone production, *Cedrus deodara*

Introduction
Conifers are one of the most ancient tree species, found from Palaeozoic era. They were widely distributed, covering most of the earth's surface at that time. This widespread distribution resulted from the spread of colonisation of new territory, from an origin probably somewhere in Asia. The massive, catastrophic evolutionary events taking place at that time wiped out a large number of conifers. Only a few species managed to adapt and survive till this date among which most of them are large, vascular, seed bearing land plants with cone like fertile structures belonging to the division Pinophyta. Majority of the plants in Pinophyta are trees and very few are shrubs, with tendency to produce seeds for hundreds or thousands of years once they attain maturity. Every year, conifers complete their reproductive cycle either by producing a new set of reproductive organs or they do not produce any reproductive organs at all. They tend to remain in their vegetative state only. Therefore, they have a good seed year once in every three to four years. Such disparity in the reproductive development of conifers has never been studied before. Therefore, it provides a grey area for research and *Cedrus deodara* has been chosen as a potential tree to carry out research in this area.
Carbohydrates and proteins are the most essential nutrients which provide energy for all types of metabolic and developmental activities. They are transported towards the developing reproductive organs at the time of cone production. The movement of stored carbohydrates and proteins from plant organs, such as leaves or roots, towards the reproductive organs

begins when fruiting commences during female meiosis (Lebon *et al*., 2000, 2008) [24, 23]. The distribution of reserved carbohydrates and proteins varies between monoecious and dioecious plants. Female plants receive a higher allocation of resources compared to male plants due to their increased reproductive activity. This preferential allocation is driven by the elevated utilization of nutrient resources in female plants for the development of reproductive organs. Consequently, this allocation results in a reduction of other growth and developmental activities in female plants. This shows that in dioecious woody plants, male show higher growth rate and survival in harsh conditions then female plants (Obeso, 2002; Nunez et. al., 2008) [25, 26]. In monoecous plants also, the plant shows higher growth rate during the development of male cones and lower growth rate during the development of female cones. Earlier works by Mathews, 1961 [27], Holmsgaard, 1986 [28], Dickmann, 1970 [27] and Kozlowski, 1968 [31] showed that developing reproductive organs act as sinks and mobilize nutrients like carbohydrates and proteins at the expense of vegetative growth of the plant. A hypothesis was put forward by Owen, 1969 [32] and Ebell, 1971 [6], that periodic cone production in conifers may be the result of utilization and draining of carbohydrate and protein reserves by the reproductive organs during a good seed year. This drain of nutrients by reproductive structures during a good seed year reduces the nutrient supply for the vegetative growth of the plant which in turn reduces the metabolic substrate and energy supply available for a continued bud development (Ebell, 1971) [6]. This may reduce the number of potentially reproductive buds which become latent or abort due to less energy supply resulting in cone crop failure the following year. *Cedrus deodara* produces cones periodically and also there is an uncertainty in its expression of dioecious and monoecious behaviour. This research investigates the influence of nutrient content on the evolving reproductive behavior of *Cedrus deodara*, aiming to elucidate the uncertainty in the expression of sexuality in conifers.

Materials and Methods

To determine the effect of carbohydrates and proteins in trees, the reproductive behaviour of *Cedurs deodara* was studied for which 300 mature trees of flowering/fruiting age were marked at three sites, *viz*, cheog forest and Himalayan Forest Research Institute (HFRI) campus forest at Panthaghati Shimla in Himachal Pradesh and Kanasar Forest, Chakrata in Uttarakhand. Each tree was observed for its reproductive behaviour and recorded as male, female, monoecious and neutral trees depending on the basis of the appearance of male cones or female cones on a single tree or both on the same tree or no cones on the single tree. The reproductive behaviour of all the trees was continuously recorded for 3 years from 2014-2016. To understand the effect of carbohydrates and proteins responsible for the unpredictable reproductive behaviour of *Cedrus deodara*, four different types of samples were collected from all the three sites. Samples from the tip of axilary branches of male, female, monoecious and vegetative plants were collected, lyophilised, powdered and preserved at -20 °C temperature. Extraction of carbohydrates and proteins was done for further estimation.

Methodology for Carbohydrate extraction and estimation

Carbohydrate extractives were extracted from wood samples by alkaline method and estimation was done by using Phenol-sulphuric acid method (Masuko T. *et al*., 2005) [11]. The extraction method used as described below was modified and standardized to extract carbohydrates from wood samples. For extraction of polysaccharides, 4 grams of each of the powdered samples were dipped into alkaline solution of 0.5 M to 1 M NaOH (Sodium Hydroxide) solution and kept at 121 °C in the hot air oven for 4 hours. Solution mixture obtained was cooled at room temperature and filtered with Whatmann filter paper. The residue left was discarded. The pH of solution obtained was neutralized by adding 1M HCl (Hydrochloric acid) solution. 200ml of ethanol was added in the neutralized solution of carbohydrate extract and left overnight at room temperature. After 24 hrs precipitate was observed at the bottom of the container. To separate the precipitate from the solution, centrifugation was done at 15000 rpm for 15 min. Pellet obtained was dissolved in water and the suspension was discarded. The solution was vacuum dried in rotary evaporator at 60 °C in round bottom flask. A dried carbohydrate extract was obtained.

1 ml of working standard of glucose from 0.2, 0.4, 0.6, 0.8 and 1ml and 0.1 to 1ml of sample dilutions were pipette out into test tubes. 1ml of volume was made up with distilled water. 1ml of Phenol solution was added in each test tube, incubated for 10 min at room temperature, 5ml of 96% of sulphuric acid was added and all the test tubes were vortexed well. All the test tubes were placed in water bath at 25-30 °C and incubated for 20 min. The absorbance was read at 490 nm with the help of UV-Vis Spectrophotometer. The amount of total carbohydrate present in the samples was calculated using the standard graph (Figure 1.)

Calculation formulae

$$\text{Total carbohydrate in the sample (\%)} = \frac{\text{Sugar value from graph } (\mu g)}{\text{Aliquot sample}} \text{ X } \frac{\text{Total volume of extract}}{\text{Wt. of sample (100mg)}} \text{ X } 1/100$$

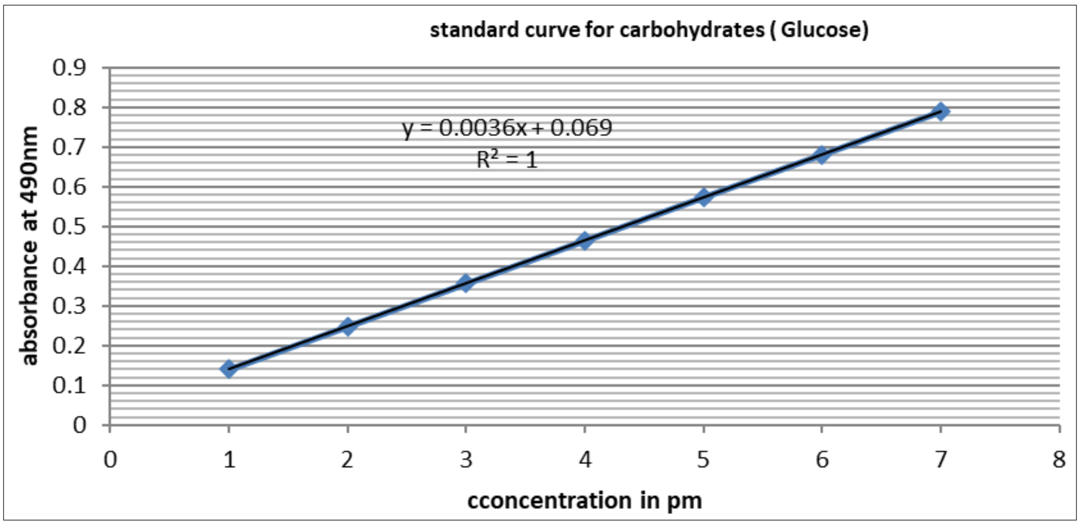


Fig 1: Standard curve for carbohydrates by using glucose as standard

Methodology for protein extraction and estimation

For extraction, isolation and estimation of proteins the following methods were studied and modified. Frozen plant material was grinded to a fine powder using mortar and pestle pre-chilled to -186 °C in liquid nitrogen. The frozen sample was transferred to ice cold extraction buffer (Phosphate buffer ((Sodium dihydrogen phosphate solution (8.954gm in 100ml of distilled water) mixed with Potassium dihydrogen phosphate solution (3.4023 gm in 100 ml of distilled water)), 10% to 20% glycerol, 1% Triton-x (v/v), 5% (w/w) PVP (polyvinylchloride), 2 mM β-mercaptoethanol) mixed well and grinded in chilled mortar and pestle. Filtered and centrifuged at 30,000rpm for 10min at 4 °C. Supernatant was stored in the conical flasks at 4 °C. A series of working standards (BSA) 0, 0.2, 0.4, 0.6, 0.8 and 1ml and series of samples from 2ppm to 100ppm were pipette out and estimated by using Lowry's method. Absorbance was read at 660 nm. Standard graph (Figure 2.) was drawn and the amount of protein was calculated in the samples.

Calculation formulae

$$\text{Total protein in the sample} = \frac{\text{Protein value from graph } (\mu g)}{\text{Aliquot sample Used } (0.1 \text{ or } 0.2 \text{ ml})} \text{ X } \frac{\text{Total volume of extract}}{\text{Wt. of sample } (100mg)} \text{ X } 1/100$$

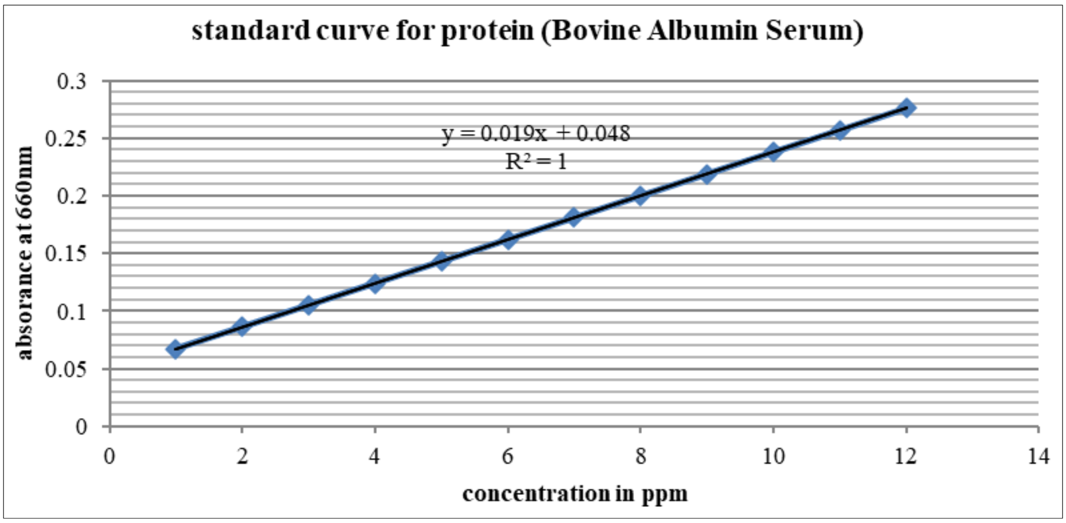


Fig 2: Standard curve for protein using Bovine Albumin Serum as standard

3. Results

Reproductive behaviour of *Cedrus deodara* was studied continuously for three years from 2014 to 2016. The flowering season starts from mid-June to the beginning of July. Male cones start appearing first. They mature at the time of pollination near the end of August. Female cones start appearing at the tip of the dwarf shoots near the end of August or the beginning of September. Pollination starts in the middle of October and ends in the middle of November.

Trees bearing male cones continuously for three years of observation were recorded and marked as male trees. Trees bearing female cones for each year of observation were marked and recorded as female trees. Trees bearing both male and female cones were marked and recorded as monoecious trees. Trees which did not show any reproductive growth for all the three years were marked and recorded as neutral trees.

Year wise percentage change in the reproductive behavior of *Cedrus deodara*

Table 1: Year wise percentage change in the number of trees at HFRI Campus forest, Cheog forest and Kanasar feorest, Chakarata.

Types of trees	HFRI campus Forest			Cheog Forest			Kanasar Forest		
	2014	2015	2016	2014	2015	2016	2014	2015	2016
%M	27	19.6	24.6	24.3	25.3	22.3	16.6	22	17.3
%F	25.6	30.6	19.1	24.3	54.6	17.3	2.6	36.3	4.6
%M/F	26.6	35.6	51	32	13.6	56.3	1.3	10.3	76.6
N%	20.6	14.0	5.3	19.3	6	4	79.3	31.3	1.3

The perusal of table 1 shows that the number of male, female, monoecious and neutral trees is not same and has changed from year to year. The number of male trees increased in the first year and then it decreased in the third year at all sites. The same happened with female trees. The number of monoecious trees increased at all three sites in the third year. Interestingly the number of neutral trees was high in the first and it decreased drastically in the third year at all three sites. A change in the number of male, female, monoecious and neutral trees was observed from 2014 to 2016 which is represented as percentage change in Table 1. The percentage change in the number of trees at all the three sites from 2014 to 2016 gives an idea about the changing reproductive pattern in *Cedrus deodara.*

Percentage change in Reproductive behavior of *Cedrus deodara* at site 1 (HFRI Campus Forest): Out of 300 trees marked at site 1 there was a remarkable change in the percentage of male, female, monoecious and neutral trees from 2014 to 2016. Change in the percentage of trees at site 1 is shown in figure 3. The Percentage of Male trees was 27% during 2014, which decreased to 19.6% in 2015 and again in 2016 it slightly increased to 24.6% but was less than the first year. In the same way the percentage of female trees in the year 2014 was 25.6% which increased to 30.6% during 2015 and again decreased to 19.1% in the year 2016. The monoecious trees increased from 26.6% and 35.6% in the year 2014 and 2015 to 51% in the year 2016. As the number of male, female and monoecious trees have changed, there has been a drastic decrease in the number of neural trees, which did not show any reproductive growth, from 20.6% in the year 2014 and 14.0% in the year 2015 to 5.3% in the year 2016.

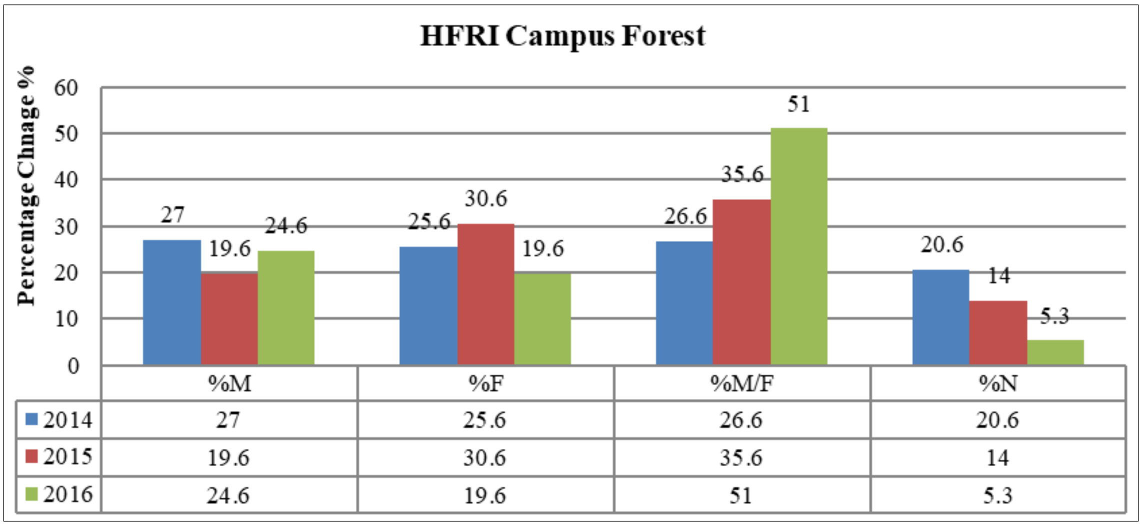
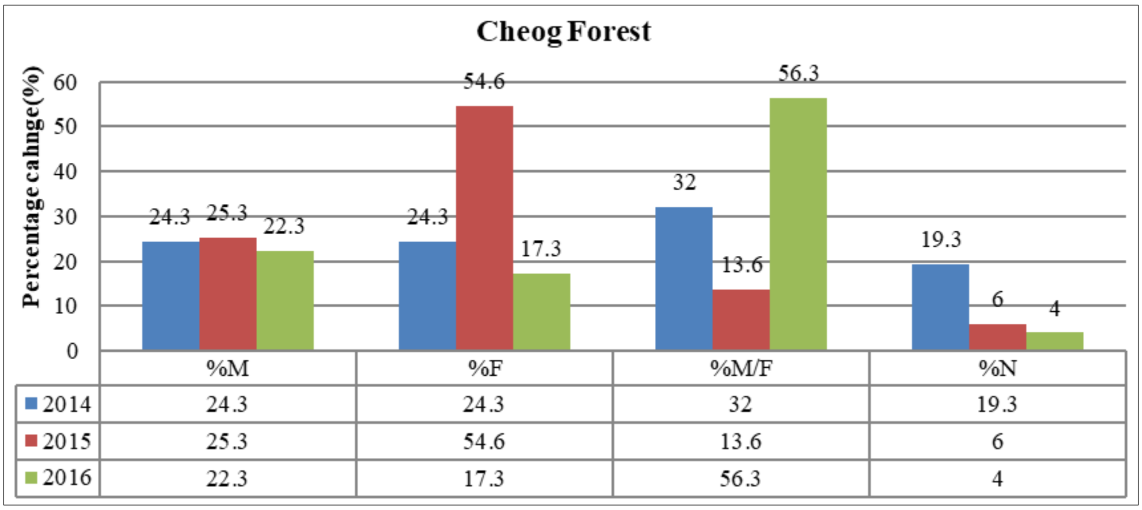


Fig 3: Percentage change in reproductive behavior of *Cedrus deodara* at site 1 (HRI campus forest)

Percentage change in the reproductive behavior of *Cedrus deodara* at site 2 (Cheog Forest)

Site 2 witnessed the same trend in the percentage change of reproductive behavior of *Cedrus deodara*. Change in the percentage of trees at site 2 is shown in figure 4. Out of 300 trees marked, the percentage of male trees was 24.3% in 2014 which increased to 25.3% during 2015 and declined to 22.3% in 2016 but was less than the first year. In the same way the percentage of female trees in the year 2014 was 24.3% which increased to 54.6% during 2015 and again decreased to 17.3% in the year 2016. Therefore a decrease in the percentage of female trees was observed at site 2 as well. The percentage of monoecious trees changed from 32% in 2014 and 13.6% in 2015 to 56.3% in 2016. Hence the number of monoecious trees increased from 32% to 56.3%. Surprisingly the percentage of neutral trees, drastically decreased from19.3% to4% from 2104 to 2016 as compared to other trees in the region.

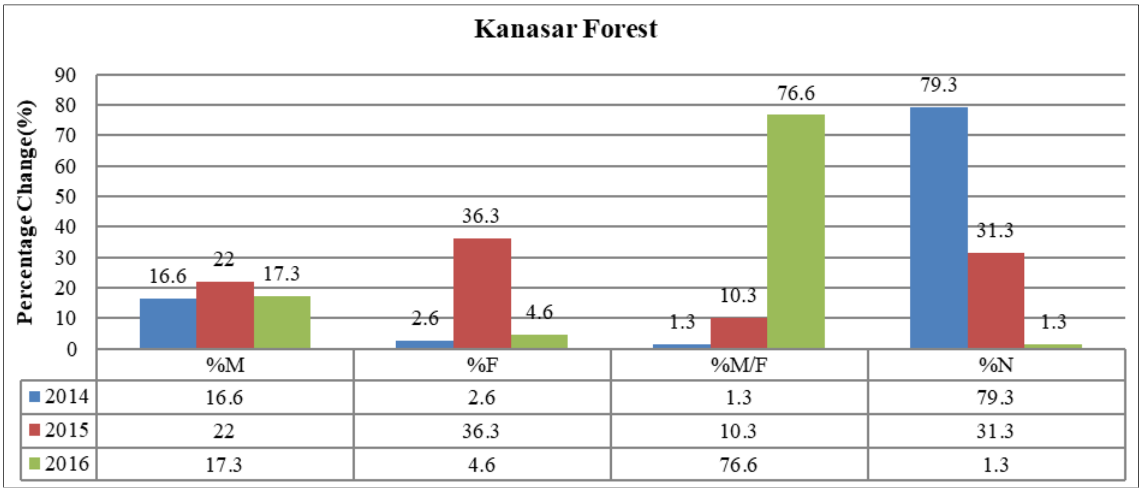


	%M	%F	%M/F	%N
2014	24.3	24.3	32	19.3
2015	25.3	54.6	13.6	6
2016	22.3	17.3	56.3	4

Fig 4: Percentage change in the reproductive activity of *Cedrus deodara* at site 2 (Cheog Forest)

Percentage change in the reproductive behavior of *Cedrus deodara* at site 3 (Kanasar Forest)

Site 3 showed slight difference in the reproductive behavior compared to site 1 and site 2. Change in the percentage of trees at site 3 is shown in figure 5. The percentage of male trees was 16.6% in 2014 which increased to 22% in 2015 and declined to 17.3% in 2016. In the same way the percentage of female trees in the year 2014 was 2.6% which increased to 36.3% in 2015 and decreased to 4.6% in 2016. Therefore a decrease in the percentage of female trees was observed at site 3. The percentage of monoecious trees has changed from 1.3% in 2014 and 10.3% in 2015 to 76.6% in 2016. Neutral trees, like other sites, has shown a drastic decline i.e., from 79.3% in the year2014 and 31.3% in the year 2015 to 1.3% in the year 2016.



	%M	%F	%M/F	%N
2014	16.6	2.6	1.3	79.3
2015	22	36.3	10.3	31.3
2016	17.3	4.6	76.6	1.3

Fig 5: Percentage change in the reproductive activity of *Cedrus deodara* at site 3 (Kanasar Forest, Chakrata)

Nutrition level in male, female, monoecious and neutral trees was checked by estimating total carbohydrate and total protein content for 2015 and 2016.

Total Carbohydrate content obtained in all four types of samples collected from each site in the year 2015

Total Carbohydrate content obtained in all four types of samples collected from each site in the year 2015 is represented in figure 6. Total carbohydrate content in samples obtained from Kanasar, Chakrata, Uttarakhand was found to be maximum in female plants followed by neutral plants and monoecious plants in order. Minimum carbohydrate content was found in male plants. Total carbohydrate content in the samples from HFRI Campus forest showed a different pattern as the total carbohydrate content was found to be maximum in monoecious plants followed by female plants and male plants in order. The minimum total carbohydrate content was found in neutral plants. The total carbohydrate content in samples from Cheog forest was found maximum in female plants followed by male plants and monoecious plants in order. The total carbohydrate content was found minimum in neutral plants.

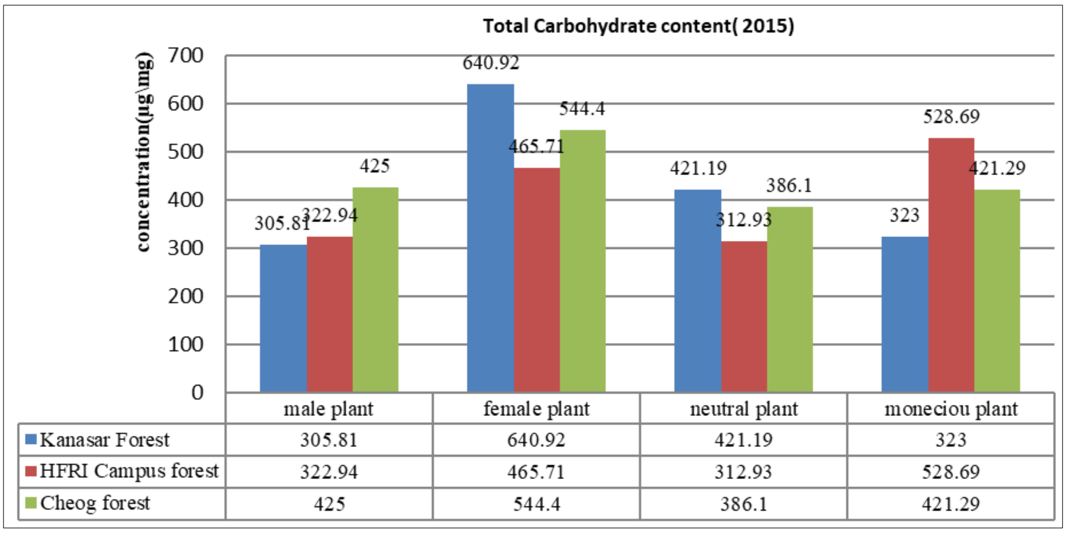


Fig 6: Total carbohydrate content in samples collected from all the three sites during 2015

Total Carbohydrate content obtained in all four samples collected from all three sites in the year 2016

Total carbohydrate content in samples from Kanasar, Chakrata, Uttarakhand was found to be maximum in monoecious plants followed by neutral plant and female plant in order as shown in the figure 7. Minimum carbohydrate content was found in male plants. Total carbohydrate content in the samples from HFRI Campus forest showed a different pattern as the total carbohydrate content was found to be maximum in neutral Plants followed by female plant and male plants in order. The minimum total carbohydrate content was found in monoecious plants. The total carbohydrate content in samples from Cheog forest was found maximum in female plants followed by monoecious plants and male plants in order. The total carbohydrate content was found minimum in neutral plants.

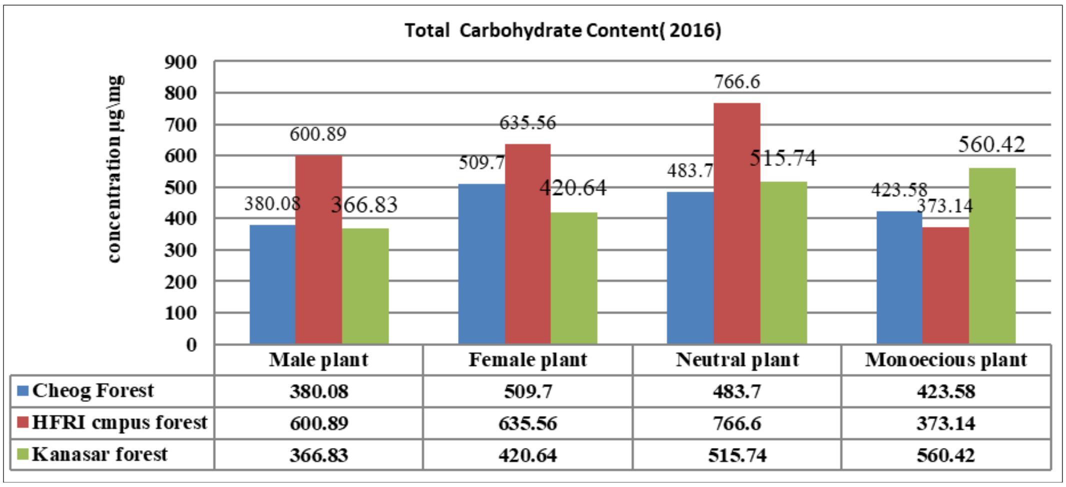
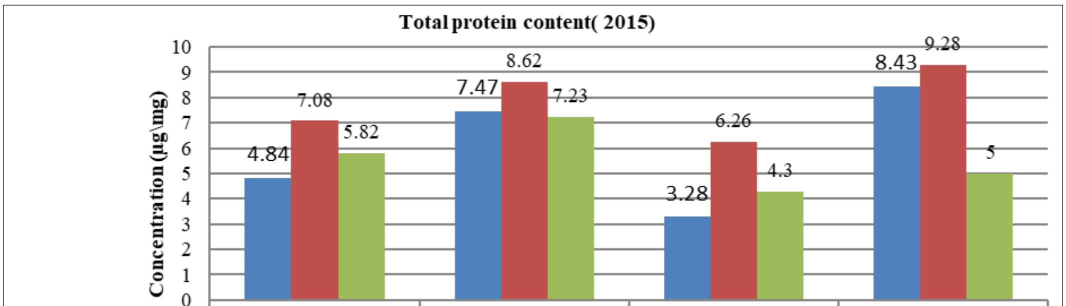


Fig 7: Total carbohydrate content in samples collected from all the three sites during 2016

Total Protein content obtained in all four samples collected from all three sites in the year 2015

Total protein content in samples collected from all the three sites *viz*, Kanasar forest, Chakrata, Uttarakhand, HFRI campus and Cheog Forest, Himachal Pradesh, for 2015, respectively is represented by figure 8. In HFRI Campus the maximum Protein content was found in monoecious plants followed by female plants and male plants in order. The Minimum protein content was found in neutral plants. In samples from Kanasar Forest, Chakrata, Uttarakhand, the maximum protein content was found in monoecious plants followed by female plants and male plants in order. The minimum total protein content was found in neutral plants. The Total Protein content in the samples collected from Cheog forest showed maximum total protein content in female plants followed by male plants and monoecious plants in order. The total protein content was found minimum in neutral plants.

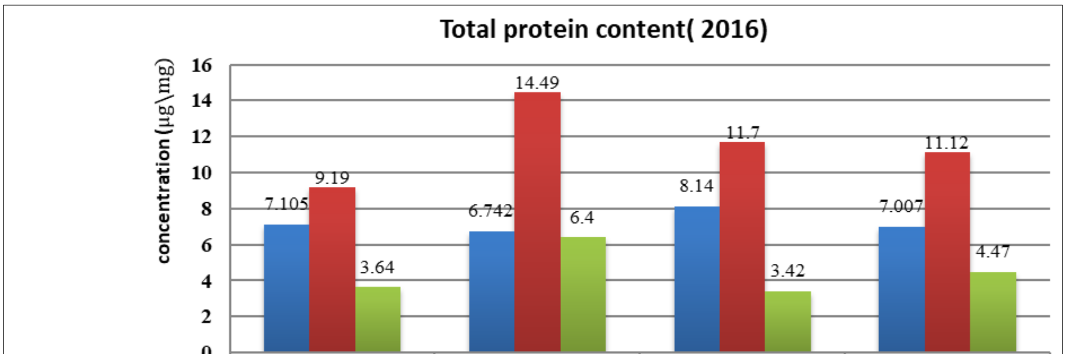


	male plant	female plant	neutral plant	moneciou plant
Kanasar forest	4.84	7.47	3.28	8.43
HFRI campus forest	7.08	8.62	6.26	9.28
Cheog forest	5.82	7.23	4.3	5

Fig 8: Total protein content in samples collected from all the three sites during 2015

Total Protein content obtained in all four samples collected from all three sites in the year 2016

Total protein content in samples collected from all the three sites *viz*, Kanasar forest, Chakrata, Uttarakhand, HFRI campus and Cheog Forest, Himachal Pradesh, respectively is represented by figure 9. In HFRI Campus the maximum Protein content was found in female Plants followed by neutral plants and monoecious plants in order. The Minimum protein content was found in male plants. In samples from Kanasar Forest, Chakrata, Uttarakhand, the maximum protein content was found in neutral plants followed by male plants and monoecious plants in order. The minimum total protein content was found in female plants. The Total Protein content in the samples collected from Cheog forest showed maximum total protein content in female plants followed by monoecious plants and male plants in order. The total protein content was found minimum in neutral plants.



	Male plant	Female plant	Neutal plant	Monoecious plant
kansar Forest	7.105	6.742	8.14	7.007
HFRI campus Forest	9.19	14.49	11.7	11.12
cheog Foest	3.64	6.4	3.42	4.47

Fig 9: Total protein content in samples collected from all the three sites during 2016

Discussion

A good seed year puts a strain on the availability of stored nutrient resources such as carbohydrates and proteins for growth and development of the tree, due to which reproductive growth of the trees is suppressed and hence, reduction in the number of seeds is observed the next year. Development of female reproductive organs require higher nutrient supply as compared to male reproductive organs, this is the reason why if a year has a greater number of female trees and monoecious trees, a decrease in the number of reproductive trees will be observed in the next year. Events in reproductive behaviour of *Cedrus deodara*, like dioecious trees turning into monoecious or monoecious trees turning into dioecious, or trees remaining in their vegetative phase for their entire reproductive cycle are highly influenced by physiological changes and nutrient availability to the trees during reproduction.

Each nutrient plays a different role in plant growth and development. Carbohydrates and proteins are the richest source of carbon and nitrogen, which are essential in various structural, metabolic and physiological functions in the tree. The utilization of nutrient reserve by monoecious and dioecious trees is different. Female trees are reproductively more active than male trees hence, allocation and utilization of nutrient reserves is more in female trees as compared to male trees (Dickmann and Kozlowski, 1968) [30]. And the allocation of nutrient reserves by monoecious trees is higher than either the male trees or female trees as the work done by monoecious trees is more compared to dioecious trees. The total carbohydrate content obtained in all the four types of trees samples i.e., male trees, female trees, monoecious trees and neutral trees, collected from HFRI campus Forest, Kanasar Forest and Cheog Forest in 2015 and 2016, was directly proportional to the change in the percentage of cone production each year. Similar was the case with the protein content obtained in all the samples in 2015 and 2016. The total carbohydrate and protein content in a sample is the measure of energy and proteins utilized at the time of cone production by the tree.

Substantial increase in the number of monoecious trees at all three sites of study and decrease in the number of neutral trees (Table 1), indicates an increase in the reproductive activity of the plants from 2014 to 2015 and 2015 to 2016. Most of the neutral trees have undergone change in their reproductive state, from vegetative phase to reproductive phase indicating a good seed year for deodar in 2016. Increase in the number of female cones in 2015 at HFRI campus forest, Cheog Forest and Kanasar forest and drop in 2016 and drastic increase in the number of monoecious trees from 2015 to 2016, points at two possibilities. First, the reproductive activity of female trees may have increased from 2014 to 2015 and decreased from 2015 to 2016 and second, most of the female trees may have turned into monoecious trees in 2016. Such change in the reproductive behavior of females indicates their higher reproductive activity. Same is with male plants in case their number increases or decreases from 2014 to 2016.

Conclusion

A good seed year puts a strain on the availability of stored nutrient resources such as carbohydrates and proteins for growth and development of the tree, due to which reproductive growth of the trees is suppressed and hence, reduction in the number of seeds is observed the next year. Development of female reproductive organs require higher nutrient supply as compared to male reproductive organs, this is the reason why if a year has a greater number of female trees and monoecious trees, a decrease in the number of reproductive trees will be observed in the next year. Events in reproductive behaviour of *C. deodara*, like dioecious trees turning into monoecious or monoecious trees turning into dioecious, or trees remaining in their vegetative phase for their entire reproductive cycle are highly influenced by physiological changes and nutrient availability to the trees during reproduction.

Change in the number of monoecious and dioecious trees at a site from 2014 to 2016 indicate the change in the reproductive activity of trees of a population at a particular site. For example, number of male trees decreased and female trees increased at HFRI Campus forest, Shimla from 2014 to 2016. On the other hand, the number of monoecious trees increased from 2014 to 2016 drastically. Such change in the number of trees every year indicates that the reproductive behaviour of the trees in a population does not remain same every year. Similar changes in the number of trees from 2014 to 2016 have been observed at Cheog Forest and Kanasar Forest, Chakrata. An increase in the number of monoecious trees from 2015 to 2016, and decrease in the number of male and female trees from 2014 to 2016, points at two possibilities. Firstly, the trees were reproductively more active in 2015 and 2016 as compared to 2014 and secondly, most of the dioecious trees changed their reproductive behaviour from dioecious to monecious. Among dioecious trees most of the male trees in 2014 changed into monoecious trees in 2016 as compared to female trees. The number of male trees transitioning into monoecious trees was higher than that of the female trees. One of the main reasons behind this phenomenal change in the representation of sexual behaviour of the trees was the utilization of nutrient reserves and energy consumption in reproductive development. As it was observed that female cone production in 2014 and 2015 was higher as compared to 2016, therefore, utilization of nutrients and energy consumption in the female trees was higher as compared to other trees, which leads to less availability of nutrients for the next reproductive cycle. As male cone production decreases gradually in 2016 as compared to 2014 and 2015, the carbohydrate content obtained in 2015 in male trees at all three study sites is less than that of female trees. This indicates that the nutrient and energy consumption by male trees is less than that of female trees during reproduction. Hence, there is a possibility that the male trees may produce male cones or become monoecious in the next reproductive cycle or they conserve nutrient for more metabolic work in the next reproductive cycle. This is the reason why a greater number of male trees turn into monoecious trees as compared to the female trees.

Each nutrient plays a different role in plant growth and development. Carbohydrates and proteins are the richest source of carbon and nitrogen, which are essential in various structural, metabolic and physiological functions in the tree. The utilization of nutrient reserve by monoecious and dioecious trees is different. Female trees are reproductively more active than male trees hence, allocation and utilization of nutrient reserves is more in female trees as compared to male trees (Dickmann and Kozlowski, 1968) [30]. And the allocation of nutrient reserves by monoecious trees is higher than either the male trees or female trees as the work done by monoecious trees is more compared to dioecious trees. The total carbohydrate content obtained in all the four types of trees samples i.e., male trees, female trees, monoecious trees and neutral trees, collected from HFRI campus Forest, Kanasar Forest and Cheog Forest in 2015 and 2016, was directly proportional to the change in the percentage of cone production each year. Similar was the case with the protein content obtained in all the samples in 2015 and 2016. The total carbohydrate and protein content in a sample is the measure of energy and proteins utilized at the time of cone production by the tree.

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