Original Research Article

Effect of temperature and light on callus of critically endangered arid species *Commiphora wightii* through slow grow storage

ABSTRACT

In vitro conservation technique is one of the potential approaches to safeguard plant biodiversity. To protect the important germplasm, storage of whole plant can be challenging. A study was done to conserve the callus of *Commiphora wightii* at different temperatures i.e. 4°C, 10°C, 20°C, 30°C and 40°C for a longer period of time as well as the effect of light and dark on callus of *Commiphora wightii* was observed. The callus was initiated from immature fruits of *C. wightii* on Gamborg B5 medium supplemented with (0.5mg/l) 2, 4 D, 3% sucrose and 0.8% agar and was sub-cultured on same media at a regular interval of 30-35 days. After two passages vigorous callus growth was obtained and thus these proliferated cultures were further placed on two separate parameters which are different temperatures and dark/light experiment on hormone-free B5 medium. The morphological changes and the fresh weight of the callus cultures were analyzed. The cultures on different temperatures were stored for a period of 90 days without sub-culturing and the data was collected on every 30 days interval. It was found that at low temperature the callus survived for 90 days, whereas at optimum temperature the sub-culturing was required after a period of 45-50 days. When the effect of dark and light was studied, the survival of callus kept in dark was around 60 days higher than the callus stored in light without sub-culturing. The survival percentage reduced after a period of 100-120 days for the cultures kept at dark.

Keywords: *Commiphora wightii*, callus cultures, temperature, light

Key message: lowering the temperature to 10°C and complete darkness can increase the storage period of *Commiphora wightii* callus for approximately 90 days and 120 days simultaneously.

1. INTRODUCTION

Commiphora wightii is a valuable medicinal plant (Chakravarty H. L., 1975) belonging to the family Burseraceae and is a native to arid regions of India. It has been extensively used in traditional medicine for its anti-inflammatory (Gupta et al., 1974), anti-arthritic (Urizar et al., 2003, Wang et al., 2004,) and hypolipidemic (Singh et al., 1994) properties. Habitat destruction and overexploitation have led to a fall in its populations, resulting in its cataloguing as critically endangered species (Ved et al., 2015). Conservation can be accomplished by *in vitro* propagation techniques which are essential for the sustainable utilization and preserving the species. *In vitro* techniques can overcome some of the issues related to plant genetic resources preservation and thus provide a promising contribution (Kulak et al., 2022). The conventional method of preserving micro-propagated species is the maintenance of clonal field collections which includes an enormous number of accessions exhibiting a wide range of genetic diversity (Shii et al, 1994, Reed B. M., 1999). *In vitro* propagation in *C. wightii* was attempted through somatic embryogenesis and organogenesis methods by numerous researchers. Through axillary shoot proliferation from seedling explants (Yusuf et al., 1999, Kant et al., 2010), internodes (Barve and Mehta, 1993, Soni V., 2010, Parmar and Kant, 2012), internodes, shoot tips and leaves (Singh et al ., 2010),

organogenesis has been induced by researchers. (Kumar et al., 2006) reported development of somatic embryo from immature leaf explants and zygotic embryo.

The technique used for the short and medium-term conservation of plant germplasm is named as “minimal growth storage” or “slow growth storage” (SGS) due to the moderations done in physical, nutritional or chemical parameters that limit the growth of the plantlets (Zayova et al., 2017). When low temperatures are applied instead of standard growth conditions, it may also be named as “cold storage”. SGS prolongs the subcultures timing whereas on optimum conditions, sub-culturing is required to be done at regular intervals of 3–5 weeks depending on the species. This enhances the conservation safety as a result of less intervention with the culture system and reducing the contamination risk during the subculture process. *In vitro* plants of many different species may survive in the standard SGS conditions. However, few other species may require species-specific conditions. Therefore, for insufficiently examined species, each chemical or physical factor may need to be assessed. The incubation at a lower temperature than that required for optimum growth reduces the metabolic activities, such as water loss, respiration, ethylene production etc. Reduced metabolic activities will ensure the steady preservation of callus cultures, resulting in the finite growth of the plantlets (Rajasekharan et al., 2015). Callus induction is a major step in tissue culture, serves as a plausible approach for mass propagation of *Commiphora wightii*. This study aims to investigate the effects of temperature and light on callus formation in *Commiphora wightii*, which can serve as a precursor to successful slow growth storage and thus reducing the sub-culturing cycles.

2. MATERIALS AND METHOD

2.1 Plant Material

2.1.1 Collection

The immature fruits, internodes, juvenile and mature leaves were collected from a disease-free mature plant of *Commiphora wightii* from field of Arid Forest Research Institute, Jodhpur (Rajasthan, India). The explant collection was done in the month of September and October.

2.1.2 Surface Sterilization

Each of these explants were initially rinsed thoroughly under running tap water for 10 min’s in a 250ml conical flask to remove dirt and debris, followed by a wash with 3-4 drops of Tween 80 (HiMedia) detergent in 200ml distilled water, later washed with distilled water 3-4 times. They were further disinfected by gentle shaking in solution of 200ml with 200mg streptomycin (HiMedia) and bavistin of each for 15 min’s followed by 3 times wash of distilled water for 2 min’s each. The surface sterilization was carried inside the Laminar Air Flow with 0.1% mercuric chloride (Sigma Aldrich) for 3-5 min’s (i.e. different type of explant treated for different time duration) followed with 3-4 times wash with sterilized distilled water for 2 min’s each to remove residual sterilants. Sterilized explants were then aseptically dissected into smaller sections and zygotic embryos were scooped out of the immature fruits.

2.1.3 Media Preparation and Autoclaving

The B5 media was prepared with the help of stock solutions of all macro, micro nutrients and vitamins (Gamborg B5, 1968) supplemented with (0.5mg/l) 2,4D and (30gm/l) sucrose as per the optimized protocol of Parmar and Kant, 2014. The pH of the media was adjusted to 5.8±0.25 with 1N NaOH/1N HCl before adding 0.8% (i.e. 8gm/l) agar and autoclaved it at 15psi, 121ºC for 15 mins.

2.1.4 Callus initiation

The callus initiated on the above described media. These cultures were incubated in controlled conditions which are 16hr/8hr of light/dark photoperiod maintained by florescent light at 25±2ºC. To proliferate, this callus was sub-cultured on same media. Two cycles of sub-culturing produce vigorous amount of callus.

2.2 Storage Conditions

2.2.1 Temperature treatment

The callus obtained after proliferation was sub-cultured on hormone free B5 (B5-HF) medium. Approximately 300-350mg of callus was inoculated in each 150ml conical flask with (60ml) B5-HF medium. In the first experiment 5 sets were made of 10 flasks each and individual sets were placed at 5 different temperatures (i.e. 4°, 10°, 20°, 30° and 40°C). Cultures were maintained at the specified temperatures for the duration of 90 days.

2.2.2 Light and dark treatment

The second experiment was conducted to study the effects of light and darkness when all the other parameters are as per the optimum conditions. 2 sets were made of 10 flasks each in which 1 set was kept in continuous darkness (0 hours/day) while in the other set the 16 hours light/8 hours darkness photoperiod (Cool white fluorescent bulbs were used as the light source) was given.

2.3 Statistical Analysis

2.3.1 Data Collection

Callus growth parameters were assessed at regular intervals throughout the experimental period. Parameters measured include morphology (Visual observations were made to assess the color and texture of the callus tissues), fresh weight (Callus samples were carefully removed from the culture medium and weighed immediately) and dry weight (Callus samples were dried in an oven at 70°C for around 48 hours until a constant weight was achieved) of the callus.

2.3.2 *Data Analysis*

All the experiments were setup in completely randomized design (CRD). Data were recorded and analyzed statistically to determine the effects of temperature and light on callus growth and survivability. Data obtained from callus growth measurements were subjected to statistical analysis using appropriate methods i.e. one way analysis of variance (ANOVA) and post hoc analysis by Duncan's multiple range test (DMRT). Significant differences among treatments were determined at a confidence level P≤0.05. Statistical software IBM SPSS Statistics 29.0.2.0 was used for data analysis.

3. RESULTS

3.1 Plant Material

3.1.1 Collection and Initiation

Only the immature fruits, internodes and juvenile leaves collected in the month of October respond to initiate callus in a period of 35-40 days. Immature fruits gave the best response among all the other explants. The survival percentage of the immature fruits after mercuric chloride treatment was found to be 93.34% and the callus induced was also higher i.e. 82.14% than the callus induction observed in juvenile leaves which is 38.46%. This callus obtained from immature fruits was further proliferated on same B5 media.

S.No.	Explant type	Media	Mercuric Chloride treatment time	Survival (%)	Callus Induction (%)
(a)	Immature fruits	B5 + (0.5mg/l) 2,4 D	5 min's	93.34	82.14
(b)	Juvenile Leaves	B5 + (0.5mg/l) 2,4 D	3 min's	86.67	38.46
(c)	Mature Leaves	B5 + (0.5mg/l) 2,4 D	3 min's	64.0	0.0
(d)	Internodes	B5 + (0.5mg/l) 2,4 D	4 min's	54.66	0.0

Table 1: Effect of mercuric chloride treatment on different explants of *Commiphora wightii* was observed.

The survival rate and the callus induction were calculated on the basis of following formulas given below.

$$\boldsymbol{Survival}\% = \frac{\boldsymbol{total\ number\ of\ explants\ inoculated - contatminated\ explants}}{\boldsymbol{total\ number\ of\ explants\ inoculated}} * \mathbf{100}$$

$$\boldsymbol{Callus\ Induction}\ \% = \frac{\boldsymbol{Number\ of\ plants\ generate\ callus}}{\boldsymbol{Total\ number\ of\ cultures\ inoculated}} * \mathbf{100}$$

S.No.	%Mean of Callus from immature fruits ± SE		%Mean of Callus from Juvenile leaves ± SE	
	FW	**DW**	**FW**	**DW**
(a)	77.25 **±** 8.36	16.80 **±** 0.87	34.33 **±** 4.92	13.66 **±** 0.72

Table 2: Percentage mean with standard errors of Fresh weight and Dry weight data of the callus obtained from Immature fruits and Juvenile leaves.

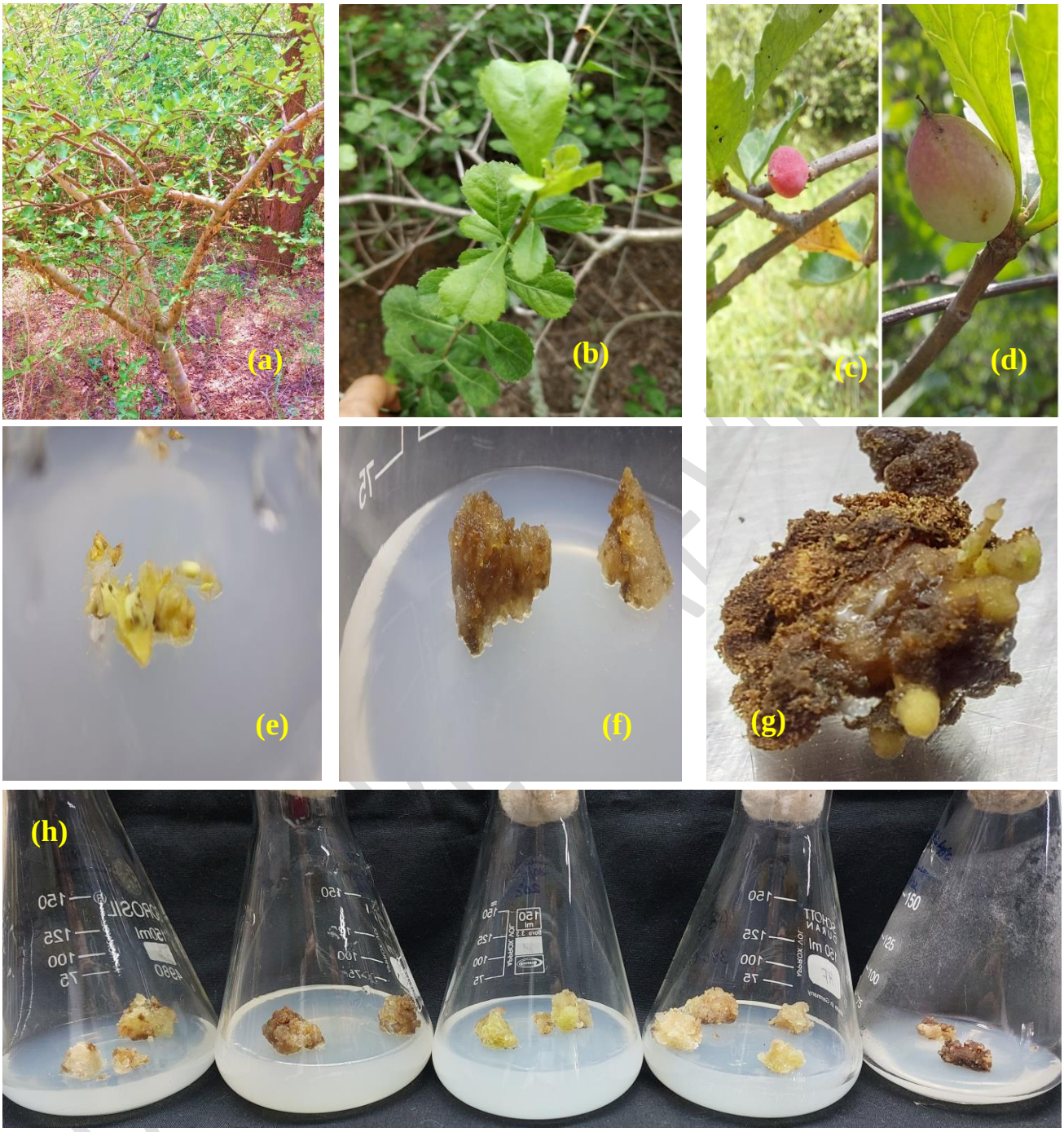

Figure 1: (a) *Commiphora wightii* tree at location *N- 26°23.079', E- 73°03.061'* AFRI, Jodhpur (b) leaves *of C. wightii* (c) Mature (pink) and (d) Immature (green) seeds of *C. wightii* collected from the plant (e) The embryos were scooped out from immature fruits and inoculated on Gamborg B5 Media with (0.5 mg/l) 2,4D (f) Callus proliferates when sub-cultured on the same media (g) The regeneration initiates in the callus on Modified MS with (0.25 mg/l) BAP, (0.1 mg/l) IBA and (0.5%) Activated Charcoal (h) The callus cultures which were stored at different temperatures i.e. 4°, 10°, 20°, 30° and 40°C (from left to right) observed after a period of 30 days.

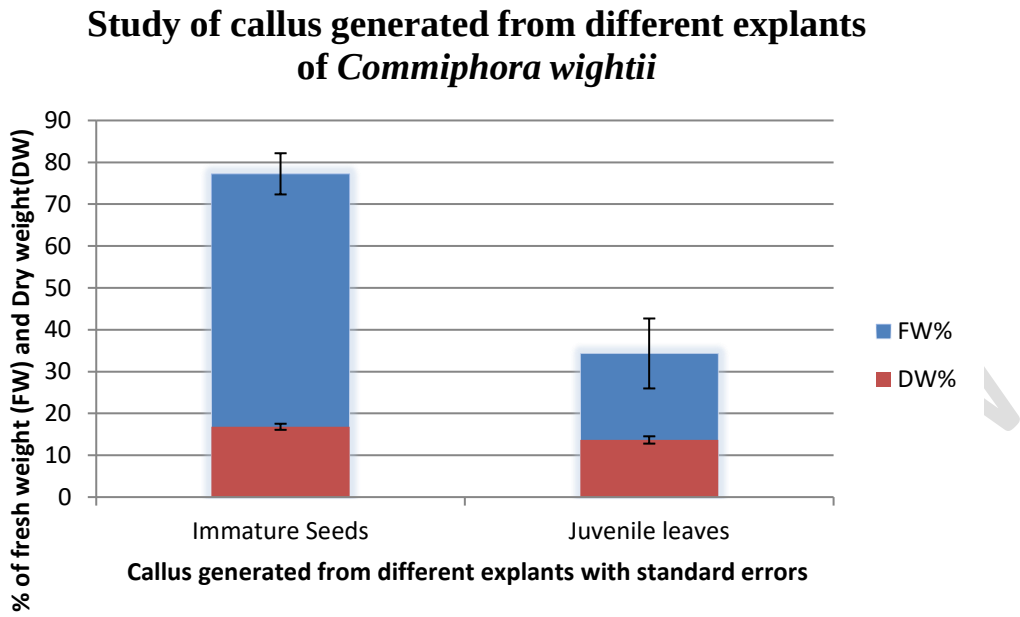

Figure 2: Depicts the moisture content (i.e. fresh weight - dry weight) and growth was high in callus obtained from immature fruits and thus this callus was used for experimental study.

The callus obtained from immature fruits is 77.25% which was higher (Figure 2) than the callus obtained from juvenile leaves i.e. 34.33% as shown in (Table 2). Thus further experiments were performed on the callus obtained from immature fruits.

3.2 Storage Conditions

3.2.1 *Temperature treatment*

Experiments were conducted to study the effect of different temperatures on the callus when stored for as long as 90 days. (Table 3) shows the callus colour and morphological effects of different temperature ranges on callus after a period of 30, 60 and 90 days.

Temperature Range (±2° C)	Number of days		
	30 days	60 days	90 days
4	Green, friable	Green, friable	Brownish yellow, friable
10	Green, friable	Brownish green, friable	Brown, friable
20	Green, friable	Greenish yellow, friable	Brownish yellow, friable Greenish brown
30	Green, friable	Greenish yellow, friable	Greenish brown, friable
40	Greenish brown, friable	Brown, compact (media depleted)	Brown, compact (The cultures do not survive)

Table 3: The morphology of the callus after following periods at different temperature ranges

It was observed (Table 3) that the callus cultures stored at 40 ± 2°C do not survive after 30-35 days as the media started to deplete and the callus turned from green and friable to brown and compact within a period of 30-35 days. Whereas, the cultures at low temperatures showed promising results as they survived for 90 days on the same media without subculturing. The green colour of the callus depicts that it is embryogenic callus. The media of cultures kept at 20°C and 30°C starts to deplete after a period of 90

days which brings the necessity of sub-culturing, whereas the callus at 4°C and 10°C survived beyond 90 days.

Table 4: The mean with standard errors of Fresh weight and Dry weight was calculated for a period of 30, 60 and 90 days at 5 different temperatures.

Temperature Range (±2°C)	Storage period (Mean±SE)					
	30 days		60 days		90 days	
	FW(gms)	DW(gms)	FW(gms)	DW(gms)	FW(gms)	DW(gms)
4	1.59± 0.16^{a}	0.37± 0.03^{a}	1.65± 0.15^{b}	0.38± 0.04^{b}	1.43±0.26^{b}	0.33± 0.04^{b}
10	1.97± 0.22^{a}	0.46± 0.05^{a}	2.22± 0.17^{c}	0.55±0.10^{bc}	2.36±0.18^{c}	0.64± 0.13^{d}
20	2.32± 0.31^{a}	0.59± 0.14^{a}	2.47±0.18^{cd}	0.68±0.10^{cd}	2.32±0.06^{c}	0.56±0.01^{cd}
30	1.85± 0.21^{a}	0.41± 0.02^{a}	2.75± 0.13^{d}	0.78± 0.05^{d}	2.06±0.16^{c}	0.43±0.02^{bc}
40	1.80± 0.24^{a}	0.38± 0.06^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00±0.00^{a}	0.00± 0.00^{a}

Mean values are calculated from three replicates. Mean±SE followed by the same alphabets, within treatments and within treatment groups do not differ significantly by DMRT test at P≤0.05.

It was observed that fresh weight of the callus kept at 4°C, 10°C, 20°C and 30°C shows (Figure 3) the increase in the growth from 30 to 60 days, while that kept at 40°C became non-viable after a period of 30-35 days. As observed the media was depleting at a faster rate at higher temperature. The Fresh weight of the callus decreased after a period of 90 days (Table 4) at temperatures 4°C, 20°C, 30°C whereas at 10°C the callus shows marginal growth. The optimum temperature for this species is around 25°C to 28°C thus growth and decline takes place exponentially as observed in callus at 30°C.

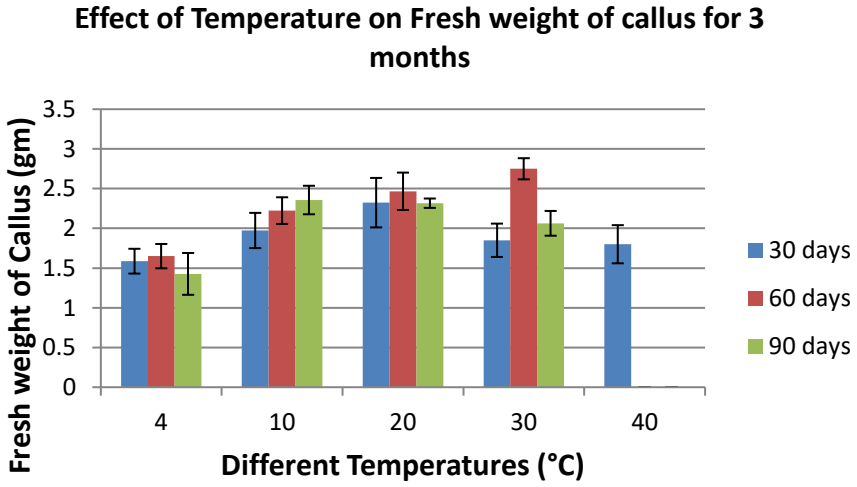

Figure 3: The fresh weight was collected after a period of 30 days, 60 days and 90 days of the callus kept at 4°C, 10°C, 20°C, 30°C and 40°C. The fresh weight of the callus was found to increase till 60 days but after which the growth decreases as can be seen on 90th day.

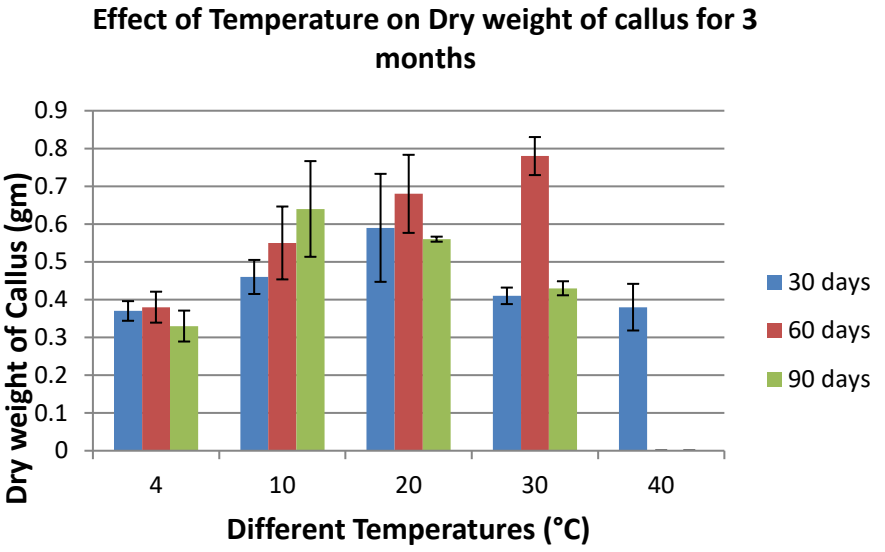

Figure 4: The dry weight was collected after a period of 30 days, 60 days and 90 days of the callus kept at 4°C, 10°C, 20°C, 30°C and 40°C. The callus collected during fresh weight data was oven dried at 70°C for a period of 48hrs.

The similar pattern was observed in the dry weight data as that of fresh weight thus it can be co-related to the above data (i.e. fresh weight data) as shown in (Figure 4).

3.2.2 Light and dark treatment

In the light and dark experiment the results obtained were as follows:

Table 5: The mean with standard error of Fresh weight and Dry weight was calculated for a period of 30, 60, 90 and 120 days at light and dark.

Experiment	Storage period	Fresh Weight (mg) (Mean±SE)	Dry Weight (mg) (Mean±SE)
Dark	**30 days**	147.93±13.23^{a}	20.83±0.50^{a}
	60 days	353.03±12.04^{b}	69.17±13.75^{b}
	90 days	481.20±36.49^{c}	110.23±9.92^{c}
	120 days	499.47±29.85^{c}	126.63±7.27^{c}
Light	**30 days**	165.93±23.36^{a}	21.73±3.58^{a}
	60 days	471.13±21.14^{c}	88.33±12.90^{ab}
	90 days	590.00±55.37^{c}	128.07±31.41^{b}
	120 days	326.37±56.57^{b}	77.30±12.55^{ab}

Mean values are calculated from three replicates. Mean±SE followed by the same alphabets, within treatments and within treatment groups do not differ significantly by DMRT test at P≤0.05.

(Table 5) shows that the growth was slow for the cultures kept in complete darkness for upto a period of 90 days i.e. 481.20mg in comparison with the callus cultures kept in 16hr/8hr photoperiod i.e. 590.00mg, while it was observed that the fresh weight of the callus kept in complete darkness stayed viable even after a period of 120 days which is 499.47mg. (Figure 5) The fresh weight of the callus is 326.37mg in 16hr/8hr photoperiod on 120th day and thus shows the decrease after a period of 90 days, it was observed that the callus had grown all over the flask and media had almost depleted. Similar results are obtained in dry weight data.

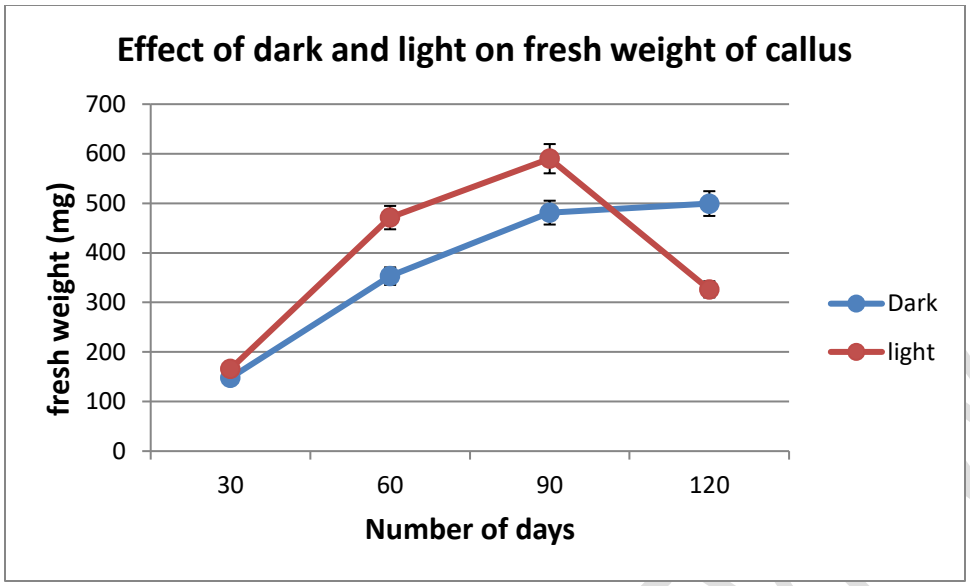

Figure 5: The fresh weight was collected after a period of 30 days, 60 days, 90 days and 120 days of the callus kept in complete darkness and 16hr/8hr photoperiod. The callus survived even after 120 days though the growth was marginal.

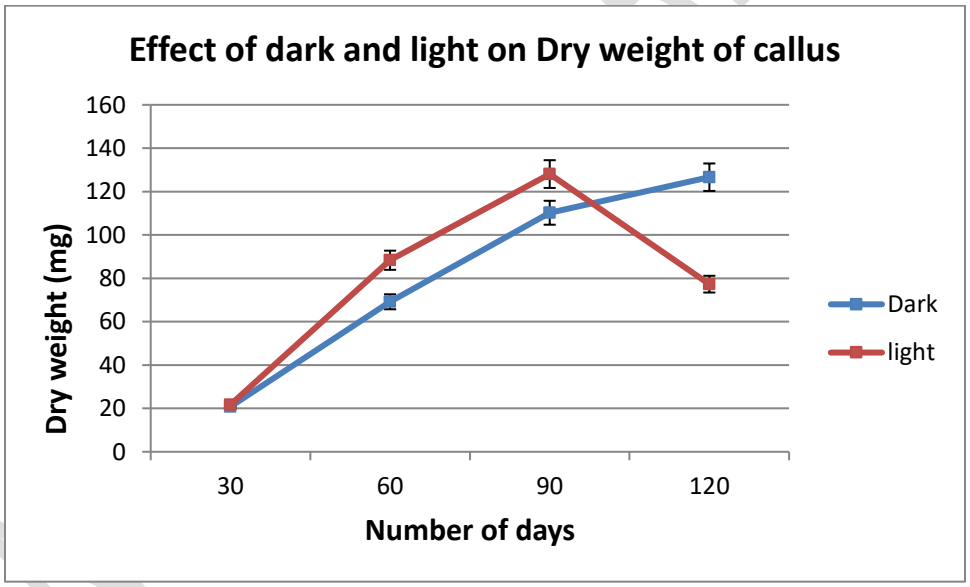

Figure 6: The dry weight was collected after a period of 30 days, 60 days, 90 days and 120 days of the callus kept in complete darkness and 16hr/8hr photoperiod. The callus collected during fresh weight data was oven dried at 70°C for a 48hrs and thus corresponding results are obtained to fresh weight.

4. DISCUSSIONS

Growth reduction is a method of slow growth storage which is generally achieved by lowering the temperature of cultures. This includes various changes in metabolic activities such as the metabolism, composition and functioning of the membranes and protein content. The aim is to increase the sub-culturing duration of the *in vitro* cultures while obtaining the regrowth after this prolonged period (Engelmann F., 1991). The cultures are usually maintained at optimum temperature, whereas for slow

growth storage the cultures are kept at lower temperatures. Some authors have reported that at low temperatures, during acclimatization of *in vitro* cultivating plants improved their survival (Malik et al., 2009, Carvalho et al., 2013). Storage of plant species at low temperatures i.e. non-freezing temperature has been successful (Koc et al., 2014). The aging of the plant cells and tissues is diminished but not entirely ceased at lower temperature regime. Eventually, successive transfer of the plant material on fresh medium is obligartory though not very regularly. In our study the 10°C temperature has been found most effective for the slow growth storage of *Commiphora wightii* calli. These results have validated the outcomes of other researchers who reported that meristem cultures of pear (Wanas et al., 1986, Bell and Reed, 2002), apple (Lundergan et al., 1979) and apple rootstocks (Orlilkowska T., 1992, Negri et al., 2000) can be stored *in vitro* at low temperatures.

In a study done by (Verma et al., 2019) the callus induction increases in *Commiphora wightii* on increasing the photoperiod from 16:8 hrs light/dark photoperiod while on decreasing the light exposure the callus growth was found to be slower. Photoreceptors sense even minute changes in light quality, which regulate the development and growth of plants through stimulating signaling pathways (Ward et al., 2005). The changes of light quality and photoperiod may instigate the regulation of photosensitive pigments on enzymes metabolizing sucrose, accelerate the activities of associated sucrose metabolizing enzymes, and make the plantlets assemble more photosynthates (Kasperbauer MJ., 2000). The need for light is not universal and varies from one species to another.

5. CONCLUSION

In vitro slow growth storage provides a plausible solution of short term and mid term storage at reduced costs and in finite spaces of the plant materials. Thus the conservation of entire plant is no more the hinderance. In this study two different methods were tried for longer storage of callus without the requirement of sub-culturing and it was found that at 10° C the callus show a marginal growth on the 90th day i.e. 2.36gm in comparison with the callus kept at all the other temperatures. The callus kept at 4° C stayed viable even after a period of 90 days but when transferred on the appropriate media for its regeneration, no response was found. In the dark and light experiments, the callus given 0 hr/day (i.e. complete darkness) of light depicts viability even after a period of 120th day, while those kept in 16/8hrs Light/Dark photoperiod shows media depletion after 90 days and thus need to be transferred on fresh medium. The outcome of the study has optimised the temperature for slow growth conservation of callus at 10° C of this critically endangered species and paves way for combined study of 10° C and dark for better results.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO use of any generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators in the writing or editing of manuscripts has been taken.

Abbreviations:

2,4D	2,4-Dichlorophenoxyacetic acid	ANOVA	Analysis of variance
CRD	Completely Randomized Design	DMRT	Duncan's Multiple Range test
HCl	Hydrochloric acid	NaOH	Sodium hydroxide
SGS	Slow Growth Storage		

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