JOURNAL OF HORTICULTURE AND POSTHARVEST RESEARCH
2024, VOL. 7(2), 197-212

Journal of Horticulture and Postharvest Research

Journal homepage: www.jhpr.birjand.ac.ir

Optimizing callus induction and analyzing *in vitro* phytochemicals in San Pedro cactus (*Echinopsis pachanoi*)

Habibeh Behnam[1], Azim Ghasemnezhad*[1], Mahdi Alizadeh[1] and Alireza Sadeghi Mahonak[2]

[1]*Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*
[2]*Faculty of Food Science & Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*

ARTICLE INFO

Original Article

Article history:
Received 22 January 2024
Revised 6 April 2024
Accepted 8 April 2024

Keywords:
Explants
Growth regulators
Tissue culture

DOI: 10.22077/jhpr.2024.7199.1359
P-ISSN: 2588-4883
E-ISSN: 2588-6169

***Corresponding author:**
Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Email: ghasemnezhad@gau.ac.ir

© This article is open access and licensed under the terms of the Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0/ which permits unrestricted, use, distribution and reproduction in any medium, or format for any purpose, even commercially provided the work is properly cited.

ABSTRACT

Purpose: The objective of the present study was to examine the impact of explant type and varying concentrations of 2,4-Dichlorophenoxyacetic acid and 6-Benzyladenine growth regulators on the San Pedro cactus callus morphological and biochemical characteristics. **Research method:** Four types of explants were used *i.e.* explants with areola, without areola, with truncated areola, and with central tissue. Additionally, five combinations of BA and 2,4-D, were tested (0 mg/L BA + 2 mg/L 2,4-D, 2 mg/L BA + 2 mg/L 2,4-D, 3 mg/L BA + 3 mg/L 2,4-D, 4 mg/L BA + 4 mg/L 2,4-D, 0 mg/L BA + 0 mg/L 2,4-D). **Findings:** The results indicated that callus formation induced in all treatments 6 days after inoculation. There were significant differences in growth parameters, including fresh weight, volume, moisture, tissue firmness, total phenols, total flavonoids and antioxidant activity of the callus ($P < 0.01$) and dry weight of callus ($P < 0.05$). Explants holding a segment of central tissue, yielded the least favorable results in most of experimental treatments, and the application of 2,4-D in the absence of BA had an inhibitory and toxic effect on the San Pedro cactus explants. **Research limitations:** No limitations were found. **Originality/Value:** Specifically, use of 2 mg/L BA + 2 mg/L 2,4-D and explants with areola resulted in callus with higher fresh weight, volume and total flavonoids, as well as good tissue integrity and firmness. The reported results are a valuable resource for future research related to cell tissue culture and the elicitation of secondary metabolites in *Echinopsis* spp.

INTRODUCTION

The San Pedro cactus, with the scientific name *Echinopsis pachanoi* is a native plant found in the southwest of America, Mexico, and Indonesia. It has been historically used for its hallucinogenic properties. Additionally, it is recognized for its diverse biological activities, including antimicrobial effects, analgesic and psychoactive properties. The cactus also contains specific biochemical compounds such as the peptide Ep-AMP1, mescaline, bridgexigenin A, B, and C, and pachanols A, B, and C (Agte et al., 1995; Kinoshita et al., 1995).

In recent years, the production of plant secondary metabolites has garnered significant attention as a potential source of effective pharmaceutical compounds (Dias et al., 2016; Hussain et al., 2012; Wang et al., 2017). The utilization of *in vitro* culture methods for extracting pharmaceutical compounds offers numerous benefits, including overcoming the limitations imposed by seasonal and geographical changes, environmental factors, and the potential for optimal and rapid production, as well as the establishment of a continuous production system in terms of quantity and quality (Rameshi, 2015). Therefore, enhancing our understanding of the growth behavior of undifferentiated cells is crucial for determining optimal subculture stages and harvesting periods for maximum biomass or metabolite accumulation (Cabanas-García et al., 2021). The literature contains limited scientific reports on the growth behavior of *in vitro* cultures of cactus species, with most focusing on *Nopalea cochenillifera* (Adki et al., 2012), cell suspension cultures of *Opuntia ficus-indica* (Llamoca-Zárate et al., 1999), *Mammillaria candida*, and *Turbinicarpus laui* (Reyes-Martínez et al., 2019). *E. pachanoi*, a medicinal cactus containing specific biochemical compounds, necessitates the study of tissue culture and callus production.

Research has shown that equal or nearly equal amounts of auxin and cytokinin can lead to favorable results in callus production. For instance, in a study on *E. chamaecereus*, treatment with 2.97 mg/L of both growth regulators BAP and NAA resulted in the highest amount of callus production. Additionally, callus formation was observed to occur more on cut surfaces (Téllez-Román et al., 2020). In another study by Angulo-Bejarano and Paredes-López (2011), it was found that among 20 combined treatments of 2,4-D and BA, the best callus induction occurred with the combination of 2.26 µM of 2,4-D and 2.21 µM of BA. Similarly, experiments on six cactus pear genotypes showed no difference among treatments in terms of the day of callus induction (Mengesha et al., 2016).

Furthermore, studies on *O.streptacantha*, *O. megacantha*, and *O. ficus-indica* revealed that the best callus production was achieved with 3 mg/L 2,4-D and 0.5 mg/L BA (Robles-Martinez et al., 2016). Notably, experiments on *Cereus peruvianus*, *Echinocactus mihanovichi*, *E. chamaecereus*, and *Aylostera heliosa* demonstrated the impact of growth regulators on callus formation (Karimi et al., 2010; Vidican et al., 2009). Additionally, hormone treatments involving 2,4-D and BAP were successfully used to induce callus in cacti such as *Notocactus magnificus* and *Curifanta macromeris* (Medeiros et al., 2006). Given the importance of the San Pedro cactus in traditional and modern medicine, the present study aimed to optimize callus induction using different explant types and plant growth regulators.
In addition, according to our literature review, no study has earlier been conducted on different explant types for callus induction in this cactus species.

MATERIALS AND METHODS

The present study was conducted as factorial experiment based on completely randomized design with four replicates. The *in vitro* studies were undertaken at the Tissue Culture Laboratory, Kesht Sanat Veshtakesht Co., situated in the Science and Technology Park of Semnan University, during the period of 2020-2021. Biochemical parameters were measured at the Horticultural Science Laboratory, Gorgan University of Agriculture and Natural Resources. The first factor was the explant type, including those containing areola (A), without areola (WA), with a cut areola (CA), and those consisting of central tissue (T). The second factor involved a combination of different concentrations of BA and 2,4-D, as detailed in Table 1.

Two-year-old San Pedro cactus plants, which have approximately 20 cm length and 5 cm diameter, were procured from the Cactus House, Semnan, Iran. To ensure uniformity in size and age, the apical part of the plants was removed. Such a treatment also can eliminate apical dominance and promote rapid meristem growth. The cactus stems were then carefully disinfected to maintain the sterility of the explants. After rinsing the stems for 30 minutes to remove any dirt or debris, the samples were treated with a 2.5% active chlorine sodium hypochlorite solution for 5 minutes to eliminate microorganisms. Subsequently, the explants were sterilized with a 70% ethanol solution for 1 minute and rinsed three times with sterile distilled water for 3, 5, and again 3 minutes, respectively.

The cactus stem was cut into 1 cm^2 explants, and three of them were placed in a jar containing Murashige and Skoog (MS) culture medium, supplemented with different hormonal concentrations, agar (8 g/l), and sucrose (30 g/l), which was then autoclaved at 121°C for 20 minutes under 1 atmosphere Pressure. Subsequently, the samples were transferred to a growth chamber with a photoperiod of 16/8 hours, and a temperature of 25±1°C. The explants growth development was monitored daily, and the explants were sub-cultured every four weeks on the same medium. After 60 days, the calli obtained from the different hormonal treatments were evaluated based on the days of callus formation, callus color, tissue firmness, callus fresh and dry weights, callus moisture percentage, callus volume, total phenols and flavonoid content, and antioxidant activity.

Measurement of total phenols

The total phenolic compounds were quantified using the Folin-Ciocalteu colorimetric method. Briefly, 0.1 g of the fresh callus tissue was ground in 5 ml of 95% ethanol and left in the dark for 24 h. Then, 1 ml of 95% ethanol was added to 1 ml of the supernatant solution, and the solution volume was adjusted to 5 ml with distilled water. Subsequently, 0.5 ml of 10% Folin reagent and 1 ml of 5% sodium carbonate were added. The resulting mixture was kept in the dark for one hour. Finally, the absorbance of each sample at a wavelength of 765 nm was measured using an Analytic Jena model spectrophotometer, and the total phenol content was calculated in mg/g fresh weight. Gallic acid concentrations ranging from 0 to 500 mg/L were used as standards. This extract was utilized to measure the total phenol content using a spectrophotometer in terms of mg/g of fresh weight (Singleton et al., 1999).

Measurement of total flavonoid content

The total flavonoid content was quantified based on the Rutin standard curve. One ml of the methanolic extract was mixed with 250 µl of 10% aluminum chloride solution and 250 µl of one-molar potassium acetate. The absorbance of the samples was measured at a wavelength of 415 nm (Akkol et al., 2008).

Table 1. Description of experimental treatments.

Hormonal treatment (mg/L)	Type of explant			
	A	WA	CA	T
A_1: 0 mg/L BA + 2 mg/L 2,4-D	A_1A	A_1WA	A_1CA	A_1T
A_2: 2 mg/L BA + 2 mg/L 2,4-D	A_2A	A_2WA	A_2CA	A_2T
A_3: 3 mg/L BA + 3 mg/L 2,4-D	A_3A	A_3WA	A_3CA	A_3T
A_4: 4 mg/L BA + 4 mg/L 2,4-D	A_4A	A_4WA	A_4CA	A_4T
A_5: 0 mg/L BA + 0 mg/L 2,4-D	A_5A	A_5WA	A_5CA	A_5T

A : explants containing areola /WA: without areola / CA: cut areola & T :central tissue

Measurement of antioxidant activity through DPPH method

The free radical inhibition was measured using the 2,2-diphenyl-2-picrylhydrazyl (DPPH) method. Initially, one ml of the methanolic extract was mixed with one ml of DPPH at a concentration of 0.1 mM. For the control, one ml of pure methanol was used as a blank. The samples were then kept in the dark for 30 minutes. Subsequently, the absorbance of the samples was measured at a wavelength of 517 nm using a spectrophotometer (Ebrahimzadeh et al., 2011).

Measurement of fresh weight, dry weight and callus moisture percentage

The callus tissue was weighed immediately after taking out of jar. Then a part of the callus was cut and after recording its weight, was transferred to an oven with a temperature of 70°C. It was remained in the oven until the weight of the sample became fixed. The difference between fresh weight and dry weight of callus was used to calculate the moisture percentage of callus (Salemlian, 2017).

Measurement of callus volume

Callus tissues after exiting from jars and weighing, were transferred to a graduated cylinder containing 20 ml of water. By calculating the difference in water volume in the cylinder before and after adding the callus, the volume of the callus was obtained (Mashayekhi & Atashi, 2018).

Data analysis

The experimental data were organized and processed using Excel, and statistical analysis was conducted using SAS software (9.1). The means were compared using Duncan's test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Initially it was found that there was no significant difference in the day of callus formation among the different treatments; all treatments-initiated callus after 6 days of cultivation (Fig. 1). Previous studies by Robles-Martinez et al. (2016) reported that callus formation from embryos culture of *O. streptacantha*, *O. Megacantha*, and *O. ficus-indica* was observed in all media supplemented with 2,4-D but not in media containing only BA. The combination of 3 mg/L 2,4-D and 0.5 mg/L BA produced the best response, with 70% of callus induction in *O. streptacantha* and 100% in *O. megacantha* and *O. ficus-indica* explants at day 15 of culture. Another experiment conducted on six cactus pear genotypes showed that there was no significant difference among genotypes with respect to time taken to callus induction (22-23.3 days after culture) (Mengesha et al., 2016).

Our findings revealed that the callus induction in San Pedro cactus is a rapid occurrence, while the maximum biomass accumulation is slow, as observed at 8 weeks after culture in the

current experiment. This behavior mirrors that of callus cultures of *Coryphantha macromeris* originating from stem discs. The highest yield of callus biomass was attained after nine weeks of culture (Cabanas-García et al., 2021). In contrast, some other plant species, such as *Eysenhardtia polystachya* (Leguminosae), demonstrated maximum biomass accumulation at 12 days of culture (Bernabe-Antonio et al., 2017), while *Armeria maritima* (Plumbaginaceae) reached maximum biomass accumulation at day 14 (Gourguillon et al., 2018).

At the end of the culture, apart from A_1 and A_5T, the callus tissue remained healthy with compact characteristics, and only a few small brown points were observed. Similar results were observed in *C. macromeris* callus after nine weeks of culture, without any phenol exodation (Cabanas-García et al., 2021). In contrast to our findings, Adki et al. (2012) reported that in *Nopalea cochenillifera* (Cactaceae) cultures, the callus lost its vigorous characteristics and turned brown 40-50 days after inoculation. Interestingly, our results demonstrate that *E.pachanoii* callus, like *C. macromeris* callus, is a long-living culture, and cells can withstand water and nutrient deficits, similar to intact- plants of cacti species (Cabanas-García et al., 2021; Stahlschmidt et al., 2011).

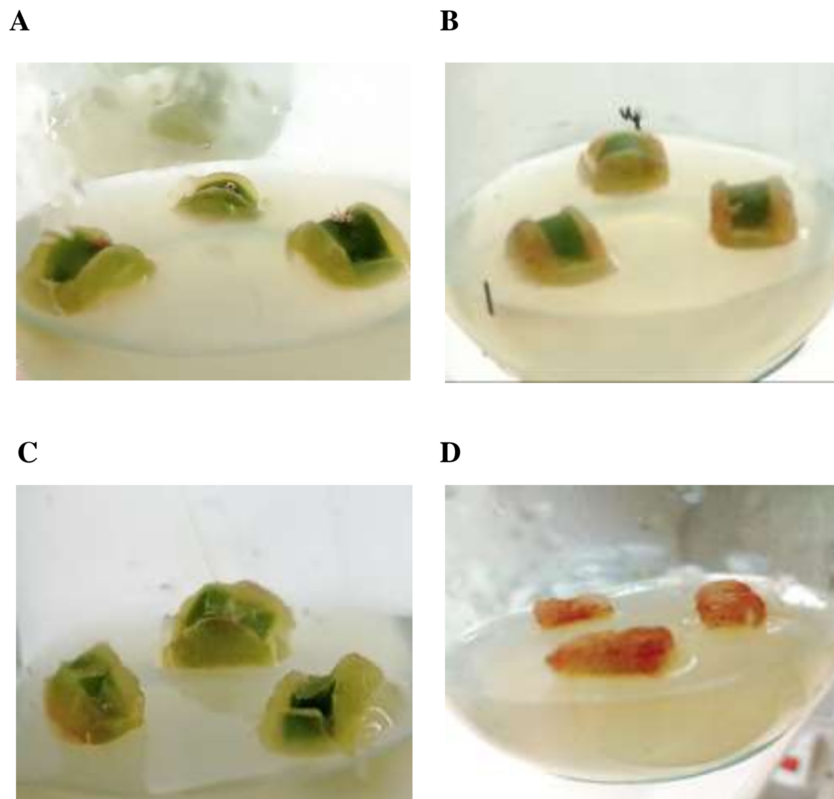

Fig. 1. Callus induction in four types of San Pedro cactus explants, 6 days after inoculation. A: explants containing an areola, B: explants without an areola, C: explants with a truncated areola, D: explants consisting of central tissue.

As far as callus color is concerned, initially the majority of calli colors were white, but as the experimental period progressed, they underwent various color changes, making it challenging to be reported as a statistically analyzed index. So, multiple colors, including white, cream, pale green, rich green, pale brown, and rich brown, were observed in all treatments, albeit in different proportions. This observation is consistent with the findings of Angulo-Bejarano and Paredes-Lopez (2011) on *Opuntia ficus-indica*, where they reported similar color changes during the development of a regeneration protocol through indirect organogenesis in the same cactus species.

The analysis of variance revealed that experimental factor, hormonal composition, has a significant impact on all studied traits ($P < 0.01$). Explant type has similar impact on studied traits ($P < 0.01$), except for total phenols and callus dry weight ($P < 0.05$) (Table 2). In Table 3, the impact of different concentrations of BA and 2,4-D on various callus morphological and biochemical growth parameters is presented. The data clearly indicates that the A_2 treatment shows the best performance in traits related to callus morphological growth, including callus fresh weight index, callus volume, and callus moisture percentage (Fig. 2). Among the experimental treatments, A_1, contained 2 mg/L 2,4-D, similar to the control (A_5), exhibited the lowest rank in fresh weight and callus volume. Additionally, the callus in this treatment displayed a brown color and had a loose and crumbling texture. Consequently, in the assessment of callus tissue firmness, treatment A_1 demonstrated the lowest value among all the experimental treatments, even lower than the control treatment. This contrasts with the findings of Teodora et al. (2015), who reported enhanced callus formation and size in *Echinopsis* (zucc) *chamaecerus* with the presence of 2.5 mg/L 2,4-D in the culture medium, compared to the control.

Table 2. The influence of explant type and combination of growth regulators on phenol, flavonoid, antioxidant activity and certain callus characteristics of San Pedro cactus.

Sources of variation	df	(Mean square)							
		Callus firmness	Fresh weight mg/ explant	Dry weight mg/ explant	Callus moisture %	Callus volume cm^3/ explant	Total phenols mg/g fw	Total flavonoids mg/g fw	Antioxidant activity %
Hormonal composition	4	13.27**	91.66**	0.21**	1458.65**	91.32**	0.58**	0.05**	512.00**
Type of explant	3	20.66**	31.55**	0.008*	1690.55**	37.31**	0.04*	0.03**	529.28**
Hormonal composition × Type of explant	19	1.87**	5.46**	0.033*	1253.02**	5.25**	0.19**	0.10**	1073.37**
Error	40	0.13	15.94	0.024	25.34	11.33	0.02	0.33	156.96
CV		10.53	17.06	8.58	5.60	14.01	20.40	19.56	20.72

ns, *, and **: No significant, significant at 5 and 1% probability, respectively.

Table 3. The effect of different concentrations of BA and 2,4-D on some callus morphological and biochemical characteristics of San Pedro cactus.

Growth regulators concentration	Callus firmness	Fresh weight mg/explant	Dry weight mg/explant	Callus moisture %	Callus volume cm^3/explant	Total phenols mg/g fw	Total flavonoids mg/g fw	Antioxidant activity %
A_1	1.91^c	0.83^c	0.04^b	94.82^a	0.88^d	0.70^b	0.44^b	58.45^b
A_2	4.16^a	6.34^a	0.27^a	95.62^a	6.50^a	1.01^a	0.55^a	56.47^b
A_3	4.08^a	5.43^b	0.22^a	95.72^a	5.83^b	0.96^a	0.48^{ab}	70.41^a
A_4	4.33^a	5.30^b	0.31^a	92.61^a	4.98^c	0.94^a	0.46^b	63.12^{ab}
A_5	2.83^b	0.57^c	0.03^b	70.20^b	0.77^d	0.49^c	0.37^c	53.78^b

Means, in each column, followed by same letter are not significantly different at the 5% probability level, using Duncan test.
A1: 0 mg/L BA + 2 mg/ l 2,4-D ،A2: 2 mg/ l BA + 2 mg/ l 2,4-D ،A3: 3 mg/ l BA + 3 mg/ l 2,4-D ،A4: 4 mg/ l BA + 4 mg/ l 2,4-D ; A5: 0 mg/ l BA + 0 mg/ l 2,4-D.

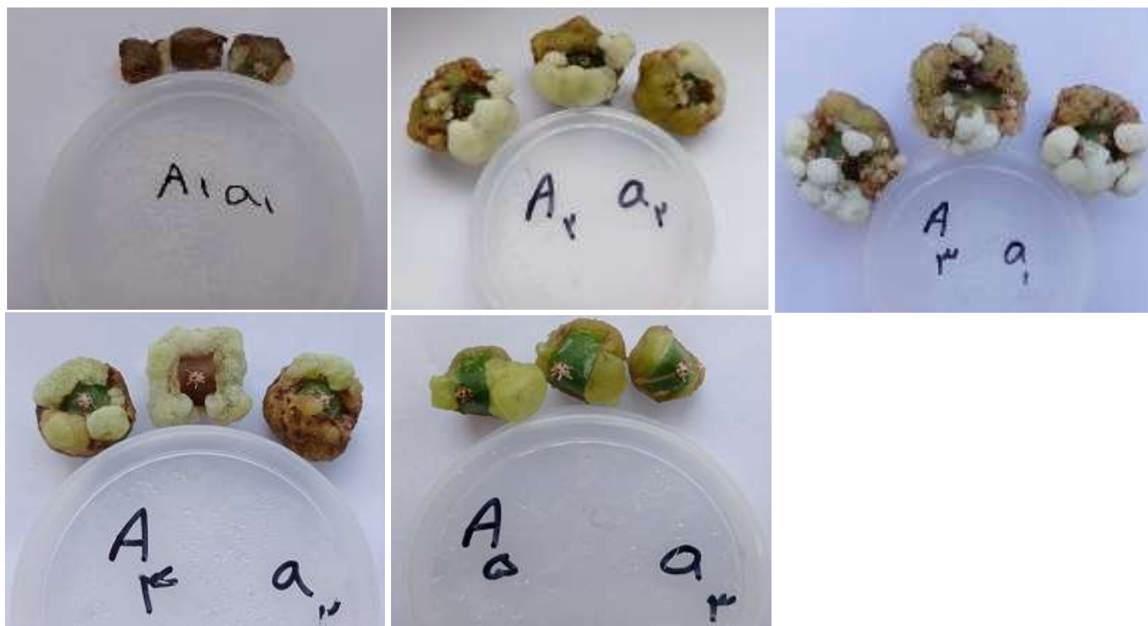

Fig. 2. Comparative callus growth of San Pedro cactus in five different concentrations of BA and 2,4-D, using explants containing areoles. A1: 0 mg/L BA + 2 mg/L 2,4-D, A2: 2 mg/L BA + 2 mg/L 2,4-D, A3: 3 mg/L BA + 3 mg/L 2,4-D, A4: 4 mg/L BA + 4 mg/L 2,4-D, A5: 0 mg/L BA + 0 mg/L 2,4-D.

The application of 2,4-D alone typically induces and promotes callus growth in most plants. However, in the case of the San Pedro cactus, the application of this growth regulator has an inhibitory effect on callus induction and growth, as evidenced by the color and tissue type of the explant. Given the reported effects associated with this growth regulator at different concentrations, it appears that cactus tissue is sensitive to 2,4-D. Therefore, it may be necessary to utilize concentrations lower than those employed in the present research. Fiedler et al. (2022) reported that the utilization of 2,4-D at moderate concentrations (2 mg/L) allowed the obtaining of a vigorous callus of *Ariocarpus retusus*, while 3 mg/L inhibited the callogenesis process.

The optimal combination of growth regulators appears to have a significant impact on increasing cell size within the callus mass. This resulted in the A_2 treatment showing higher fresh weight and volume of the callus compared to other treatments. When comparing the experimental treatments involving different concentrations of growth regulators (A_1, A_2, A_3, and A_4) to the control treatment (A_5), there was a noticeable increase in the percentage of moisture. A higher moisture content is often associated with higher cell viability (Tan et al., 2010), suggesting that growth regulators may play a crucial role in facilitating water and nutrient absorption, as well as promoting cell wall expansion within the callus mass. This observation is consistent with previous research, such as the high callus moisture content of *Grewia tenax* obtained with BA+NAA calli compared to NAA, which can be attributed to the presence of cytokinin in the culture medium (Daffalla et al., 2019). Research has shown that application of cytokinin can stimulate the growth of unorganized cultures (Luczkiewicz et al., 2014). These findings support the notion that growth regulators can have a significant impact on callus morphology and biochemical parameters.

There were significant differences in total phenols and flavonoid contents among the treatments. A_2 treatment has shown most total phenol content, however it had no significant differences with A_3 and A_4 treatments, but all of them had significant differences with A_1 and

A_5 treatments, respectively. A_2 had most total flavonoids content too. Also, notable variations were observed in antioxidant activity, with treatment A_3 exhibiting the highest level, followed by treatment A_4, and then other treatments. Importantly, the control treatment (A_5) displayed lower antioxidant activity compared to the experimental treatments containing growth regulators. Results showed that antioxidant activity in San pedro cactus may increase with higher concentrations of plant growth regulators.

Remarkably, previous studies have also shown the influence of specific growth regulator combinations on antioxidant activity in plant callus cultures. For instance, in *Salvia moorcroftiana* callus, the maximum DPPH scavenging activity was achieved using 2,4-D and BAP at 1mg/L each, along with 1.5 mg/L melatonin (Bano et al., 2022). Similarly, in *Ocimum basilicum* L. callus, the highest levels of antioxidant content were achieved with 0.5 mg/L 2,4-D (Wongsen et al., 2015). Furthermore, callus cultured on a medium supplemented with 2.0 mg/L 2,4-D and 1.0 mg/L BAP showed the greatest antiradical activities against DPPH in *Crataegus azarolus* callus (Chaâbani et al., 2015). These findings are consistent with research on *S. tebesana* callus culture, which revealed the highest antioxidant activity in callus extracts derived from shoot apical meristems on a medium with 0.5 mg/L 2,4-D and 1 mg/L BAP (Hemmati et al., 2020). Similarly, maximum DPPH free radical scavenging activity in *Cnidium officinale* was reported in callus grown on a medium supplemented with 2.3 µM 2,4-D and 2.2 µM BA (Adil et al., 2018). These collective results, along with numerous other studies, underscore the role of plant growth regulators in stimulating and enhancing antioxidant activities in plant callus cultures.

Analysis of the impact of four distinct explant types on callus induction and growth traits in Table 4 reveals that the explant prepared from central tissues, exhibits the lowest amount among all studied traits, except total phenol, significantly differing from the other three explant types. Furthermore, the comparison of biochemical traits indicates that the central tissue explant displayed lower antioxidant activity as compared to the other explants (Fig. 3).

Previous research by Elias et al. (2014) has demonstrated that skin removal or wounding on *Echinocereus cinerascens* explants leads to induce callus production. In present experiment, no difference was observed among explants containing areole and those with a truncated areole. The callus formation mainly occurred in the basal part of the explants, consistent with findings reported by Cabanas-Garcia et al. (2021) in *C. Macromeris*. However, studies by Karimi et al. (2010) on *C. jamacaru* and *C. hildmannianus*, as well as Mondragón-Jacobo and Chessa (2010) on pear cactus, suggest that the areole was not effective for callus induction. In contrast to these results, the explants containing an areole did not show any diffrence evidence of callus induction in *E. pachanoi*. Callus tissues produced in these explant types were more compact and denser than others.

Fig. 3. The *in vitro* performance of different explants of San Pedro cactus inoculated on A_2 medium 60 days after inoculation.

Table 4. The effect of explant types on some morphological and biochemical parameters of callus in San Pedro cactus.

Explant type	Callus firmness	Fresh weight mg/explant	Dry weight mg/explant	Callus moisture %	Callus volume cm^3/explant	Total phenols mg/g fw	Total flavonoids mg/g fw	Antioxidant activity %
A	4.33[a]	4.37[a]	0.19[a]	95.14[a]	4.50[a]	0.89[a]	0.43[b]	60.91[ab]
WA	3.93[b]	4.30[a]	0.18[a]	95.30[a]	4.46[a]	0.84[ab]	0.47[ab]	66.89[a]
CA	3.86[b]	4.58[a]	0.19[a]	94.85[a]	4.57[a]	0.79[ab]	0.42[b]	61.48[ab]
T	1.73[c]	1.53[b]	0.14[b]	73.87[b]	1.43[b]	0.76[b]	0.52[a]	52.50[b]

Means, in each column, followed by same letter are not significantly different at the 5% probability level, using Duncan Test. A : explants containing areola /WA: without areola / CA: cut areola & T :central tissue.

Table 5. The interaction effect of growth regulators and the explant type on some callus morphological characteristics in San Pedro cactus.

Growth regulators	Explant type	Callus firmness	Callus volume cm^3/explant	Callus moisture %	Dry weight mg/explant	Fresh weight mg/explant
0 mg/L BA + 2 mg/L 2,4-D	A	2.00[e]	0.88[i]	94.38[a]	0.04[efg]	0.81[fg]
	WA	2.00[e]	0.88[i]	94.99[a]	0.04[efg]	0.80[fg]
	CA	2.00[e]	0.88[i]	94.42[a]	0.04[efg]	0.84[fg]
	T	1.66[e]	0.88[i]	95.48[a]	0.03[efg]	0.87[fg]
2 mg/L BA + 2 mg/L 2,4-D	A	5.00[a]	7.66[b]	95.43[a]	0.33[abc]	7.24[b]
	WA	5.00[a]	7.55[bc]	95.69[a]	0.31[abcd]	7.28[b]
	CA	5.00[a]	8.66[a]	95.73[a]	0.37[ab]	8.84[a]
	T	1.66[e]	2.11[g]	95.63[a]	0.08[cdefg]	2.00[e]
3 mg/L BA + 3 mg/L 2,4-D	A	5.00[a]	7.33[bcd]	95.81[a]	0.29[abcde]	7.00[b]
	WA	4.66[ab]	7.22[bcd]	95.75[a]	0.28[abcdef]	6.81[bc]
	CA	4.33[b]	6.77[cd]	95.88[a]	0.25[bcdef]	6.25[bc]
	T	2.33[d]	2.00[gh]	95.44[a]	0.07[cdefg]	1.65[ef]
4 mg/L BA + 4 mg/L 2,4-D	A	4.66[ab]	5.44[f]	96.10[a]	0.22[bcdefg]	5.78[c]
	WA	5.00[a]	6.55[de]	95.67[a]	0.25[bcdefg]	5.84[c]
	CA	4.66[ab]	5.77[ef]	95.85[a]	0.26[bcdef]	6.46[bc]
	T	3.00[c]	2.16[g]	82.80[b]	0.52[a]	3.11[d]
0 mg/L BA + 0 mg/L 2,4-D	A	5.00[a]	1.16[hi]	94.00[a]	0.06[defg]	1.03[efg]
	WA	3.00[c]	1.16[hi]	94.40[a]	0.04[efg]	0.76[fg]
	CA	3.33[c]	0.77[ij]	92.39[a]	0.36[fg]	0.49[g]
	T	0.00[f]	0.00[j]	0.00[c]	0.00[g]	0.00[g]

Means, in each column, followed by same letter are not significantly different at the 5% probability level, using Duncan Test. A : explants containing areola /WA: without areola / CA: cut areola & T :central tissue.

The interaction effects of explant type and hormonal combination on callus were shown in Tables 5 and 6. The control treatment A_5 (0 mg/L BA + 0 mg/L 2,4-D) with the central tissue explant has been normalized to zero across all investigated parameters. Notably, after 4 weeks from the initial cultivation date, when the explants were expected to be thriving, all the explants in this experimental treatment were observed to be dark brown in color, turning black, and ultimately resulting in complete tissue death and destruction. Consequently, the cultivation of these explants was discontinued during the experiment. This occurrence suggests that the presence of growth regulators in the central tissue explant medium is crucial for sustained growth.

The results indicate that noticeable differences were observed in all growth parameters at a 5% probability level. Table 5 suggests that the central tissue explant type yielded the least favorable outcomes across all the experimental treatments. Additionally, the application of 2 mg/L 2,4-D (treatment A_1) did not have a positive effect on callus growth, resulting in a very loose, fragile, and weak callus texture, even weaker than the control treatment. This indicates that the use of the growth regulator 2,4-D in the absence of BA may induce toxicity symptoms in San Pedro cactus explants. It is worth noting that the efficiency of different combinations and concentrations of plant growth regulators to stimulate callus induction could be different depends on plant species and type of the tissue (Dawa et al., 2017).

Table 6. The interaction effect of BA and 2,4-D and the type of explants on some biochemical traits of callus in San Pedro cactus.

Growth regulators	Explant type	Antioxidant activity %	Total flavonoids mg/g fw	Total phenols mg/g fw
0 mg/L BA + 2 mg/L 2,4-D	A	46.89^{c}	0.36^{efg}	0.68^{defgh}
	WA	77.13^{a}	0.68^{ab}	0.87^{cdefg}
	CA	45.59^{c}	0.33^{g}	0.59^{h}
	T	64.21^{abc}	0.41^{defg}	0.66^{efgh}
2 mg/L BA + 2 mg/L 2,4-D	A	78.66^{a}	0.82^{a}	1.48^{a}
	WA	53.74^{bc}	0.52^{cd}	0.74^{cdefgh}
	CA	60.71^{abc}	0.43^{defg}	0.85^{cdefgh}
	T	60.71^{abc}	0.42^{defg}	0.97^{bc}
3 mg/L BA + 3 mg/L 2,4-D	A	69.83^{ab}	0.35^{fg}	0.95^{bcd}
	WA	65.03^{abc}	0.42^{defg}	0.94^{bcde}
	CA	69.20^{ab}	0.63^{bc}	0.98^{bc}
	T	77.58^{a}	0.51^{cde}	0.98^{bc}
4 mg/L BA + 4 mg/L 2,4-D	A	68.94^{ab}	0.50^{cdef}	1.18^{b}
	WA	59.89^{abc}	0.44^{defg}	0.95^{bcd}
	CA	63.65^{abc}	0.31^{g}	0.92^{bcdef}
	T	60.04^{abc}	0.61^{bc}	0.69^{defgh}
0 mg/L BA + 0 mg/L 2,4-D	A	68.19^{ab}	0.51^{cde}	0.65^{fgh}
	WA	50.73^{bc}	0.54^{cde}	0.69^{defgh}
	CA	68.27^{ab}	0.43^{defg}	0.62^{gh}
	T	0.00^{d}	0.00^{h}	0.00^{i}

Means, in each column, followed by same letter are not significantly different at the 5% probability level, using Duncan Test. A : explants containing areola /WA: without areola / CA: cut areola & T :central tissue.

The comparative analysis of the three A_2, A_3, and A_4 treatments and control (A_5), revealed that they yielded better results in terms of fresh weight, volume, and firmness of the callus tissue. Among these treatments, A_2 was found to be the most effective.

The color of callus tissue can provide valuable insights into its quality and physiological status. As it was already noted, when the callus tissue color was transitioned from dark brown to black, it was ultimately leading to tissue death and destruction. This observation underscores the potential link between callus color and its overall quality and viability. In a study on Taxus callus cultures, Wickremesinhe and Arteea (1993) reported that the callus color gradually changed from pale yellow to dark brown over a six-week period, with a subsequent decrease in the mitotic index. The absence of mitotic figures in the dark brown callus cells further supports the association between callus color and physiological changes.

In plant tissue culture, the color of callus tissue can reflect various physiological and biochemical processes within the cells. Darkening or browning of callus tissue is often associated with the accumulation of phenolic compounds, oxidative stress, and cell death. These color changes are attributed to the oxidation of phenolic compounds by enzymes such as polyphenol oxidase, resulting in the formation of dark pigments (Taghizadeh & Dastjerdi, 2020). Phenolic compounds are released as a natural defense response against plant injuries, potentially leading to damage or cell death (Amente & Chimdessa, 2021). Hesami et al. (2018) emphasized that tissue browning in tissue culturing occurs due to the accumulation and oxidation of phenolic compounds.

Furthermore, research on divided pigeon orchid (*Dendrobium crumenatum* Swartz) callus revealed that dark brown callus had significantly higher polyphenol oxidase (PPO) activity and total phenolic content as compared to green callus (Kaewubon et al., 2014). Similar findings were observed in peony tree (*Paeonia suffruticosa* Andr.) roots (Fu et al., 2011), *Pinus virginiana* Mill callus (Tang & Newton 2004), and the browning of bamboo shoots

(Huang et al., 2002). Ultrastructural disorganization involving the nucleus, mitochondria, and chloroplasts serves as indicators of enzymatic oxidative browning. Therefore, levels of PPO and total phenolics can serve as biochemical markers when selecting suitable callus (Kaewubon et al., 2014). These collective findings underscore the importance of considering callus color as a reflection of underlying physiological and biochemical changes in plant tissue culture.

The presence of dark-colored callus may indicate an imbalance in the tissue culture environment, potentially signifying stress, nutrient deficiencies, or the accumulation of toxic compounds. In an experiment on peony tree callus culture, the evidence indicated that the browning of callus is influenced by various factors, including the composition of the medium, such as macro elements of Murashige and Skoog (MS salts) and iron salt (Fe^{2+}), pH, agar, and specific plant growth regulators like 6-benzylaminopurine (6-BA), 1-naphthaleneacetic acid (NAA), and kinetin (KT). The optimal medium for preventing callus browning was found to be 1/2 MS medium supplemented with 6.95 mg/L Fe^{2+}, 0.3 mg/L KT, 0.5 mg/L NAA, 6.0 g/l agar, at a pH of 6.5 (Zhou et al., 2016).

Additionally, other research has shown that exposure of green callus of castor bean (*Ricinus communis* L.) to certain concentrations of $CuSO_4$ resulted in the callus turning brownish and eventually partly dark brown (Huang et al., 2016). Consequently, the quality of callus tissue can be compromised, affecting its suitability for subsequent stages of tissue culture, such as regeneration or organogenesis. Furthermore, the morphological characteristics and cell viability of coffee plant callus have revealed that yellow callus exhibits higher cell viability, potentially contributing to a greater potential for embryogenesis (Pádua et al., 2014). These findings underscore the importance of considering callus color as an indicator of underlying physiological and biochemical changes in plant tissue cultures, as well as its impact on subsequent developmental processes.

On the other hand, healthy and high-quality callus tissue often displays a light, creamy, or greenish coloration, indicating active growth and physiological balance. This type of callus is more likely to possess the desired characteristics for further developmental processes, such as somatic embryogenesis, shoot organogenesis, or the extraction of secondary metabolites. For instance, in the case of *Taxus*, it has been observed that pale-yellow-colored callus was selected for subculture to enhance Taxol production, while brown-colored callus was discarded due to its eventual progression to callus death (Wickremesinhe & Arteea, 1993).

Furthermore, previous research by Ashokhan et al. (2019; 2020) demonstrated that green callus contains the highest content of bioactive compounds, antioxidant and cytotoxic potentials, as well as the highest amount of Azadirachtin, an essential biopesticide in *Azadirachta indica*, compared to brown callus. These findings emphasize the significance of callus color as an indicator of its potential for further applications, such as the production of bioactive compounds and essential secondary metabolites.

Table 6 illustrates the interaction between different combinations of plant regulators and explant types on various biochemical traits of callus. Phenolic acids are molecules that contain at least one carboxylic substituent bonded to an aromatic ring (Robbins, 2003). Phenolic metabolites have garnered significant attention due to their pharmacological and functional properties, such as antioxidant, anticarcinogenic, and anti-inflammatory effects (Martinez-Valverde et al., 2000). The highest total phenol content was observed in A_2 treatment with areola explant and A_4 treatment with areola explant.

Flavonoids are polyphenolic metabolites that have drawn attention due to their health-promoting effects in diseases such as cancer, Alzheimer, and others, as well as their functional applications in cosmetic, pharmaceutical, and medicinal industries (Panche et al., 2016). In terms of the flavonoid index, A_2-A exhibited the highest flavonoids content at the

5% probability level compared to all treatments, followed by A_1-Wa, A_3-CA and A_4-T. Regarding antioxidant activity, the treatments A_1-WA, A_2-A and A_3-T demonstrated the highest levels of antioxidant activity. Conversely, the treatments A_1-A, A_1-CA, and A_5-T displayed the lowest levels of antioxidant activity, respectively.

Based on the findings, there are correlations that can be inferred regarding the quality of callus and the presence of phytochemicals under different treatments. The text highlights the impact of interactions between various combinations of plant regulators and explant types on the biochemical traits of callus. Specifically, the effects of treatments on total phenol content, flavonoids, and antioxidant activity are discussed. The highest total phenol content was observed in the A_2 treatment with areola explant and the A_3 treatment with areola explant. This suggests that specific treatments have the potential to enhance the accumulation of phenolic compounds, which are important phytochemicals associated with antioxidant and other beneficial properties. This means that higher phenolic content may indicate better callus quality in terms of its potential health-promoting properties, as phenolic compounds are generally present in healthy plant tissues (Amente & Chimdessa, 2021).

The results indicate that treatments A2-A and A_2-T exhibited the highest flavonoid accumulation. Known for their health-promoting effects, the increased presence of flavonoids may suggest improved callus quality for potential functional applications in industries such as cosmetics, pharmaceuticals, and medicine (Jedinak & Maliar, 2004). Additionally, treatments A_1-WA, A_2-A, A_3-CA and A_3-T demonstrated the highest levels of antioxidant activity. Antioxidants play a crucial role in protecting cells from damage caused by free radicals, and higher antioxidant activity in callus may indicate better quality with potential health benefits (Raj et al., 2020; Nishchal et al., 2018). It has also been observed that antioxidant compounds can influence callus growth (Huh et al., 2017). In summary, the different treatments had a significant impact on the phytochemical composition of callus, and there are correlations between the presence of specific phytochemicals and the quality of callus. These findings are valuable for understanding how different treatments can influence the accumulation of bioactive compounds in callus and may provide insights into optimizing tissue culture protocols to enhance the production of high-quality callus with desirable phytochemical profiles.

CONCLUSION

In conclusion, the results demonstrate that the A_2-A treatment yields callus with superior weight and volume, indicating its effectiveness in promoting callus growth. Moreover, the resulting callus exhibits exceptional quality characterized by its consistency and firmness, making it highly suitable for further research related to San Pedro cactus regeneration and the induction and production of hairy roots from callus. The callus obtained from this treatment displays a color spectrum ranging from white to cream and light green, further highlighting its potential for future applications. The favorable quality of the callus generated through the A_2-A treatment recommends its use in callus generation for other valuable cacti species. These findings provide valuable insights into the optimal combinations of plant regulators and explant types for callus induction in San Pedro cactus. This data can significantly contribute to the development of efficient and sustainable methods for the propagation and conservation of this valuable plant species. Furthermore, the study's findings may serve as a valuable resource for future research related to cell tissue culture and the elicitation of secondary metabolites in *Echinopsis* spp. and other cacti species. By understanding the effects of different treatments on callus quality and phytochemical composition, researchers can advance the development of innovative approaches for enhancing the production of high-

quality callus with desirable phytochemical profiles, thereby contributing to the broader field of plant tissue culture and conservation.

Conflict of interest
The authors declare that there is no conflict of interest.

REFERENCES

Adki, V.S., Jadhav, J.P., & Bapat, V.A. (2012). Exploring the phytoremediation potential of cactus (*Nopalea cochenillifera* Salm. Dyck.) cell cultures for textile dye degradation. *International Journal of Phytoremediation, 14*, 554–569. https://doi.org/10.1080/15226514.2011.619226.

Adil, M., Ren, X., Kang, D., Luc, T., & Jeong, B. R. (2018). Effect of explant type and plant growth regulators on callus induction, growth and secondary metabolites production in *Cnidium officinale Makino*. *Molecular Biology Reports, 45*(3), 1919-1927. https://doi.org/10.1007/s11033-018-4340-3.

Agte, V. V., Gokhale, M. K., Paknikar, K. M., & Cheplonkar, S. (1995). Assessment of pearl millet vs rice-based diets for bioavailability of four trace metals. *Plant Foods for Human Nutrition*, *48*(2), 149-158. https://doi.org/10.1007/BF01088311.

Akkol, E. K., Orhan, I. E., & Yeşilada, E. (2012). Anticholinesterase and antioxidant effects of the ethanol extract, ethanol fractions and isolated flavonoids from *Cistus laurifolius* L. leaves. *Food Chemistry, 131*(2), 626-631. https://doi.org/10.1016/j.foodchem.2011.09.041

Amente. G. & Chimdessa. E. (2021). Control of browning in plant tissue culture: A review. *Journal of Scientific Agriculture, 5*, 67-71. https://doi.org/10.25081/jsa.2021.v5.7266.

Angulo-Bejarano, P. I., & Paredes-López, O. (2011). Development of a regeneration protocol through indirect organogenesis in prickly pear cactus (*Opuntia ficus*-Indica (L.) Mill). *Scientia Horticulturae, 128*(3), 283-288. https://doi.org/10.1016/j.scienta.2011.01.030.

Ashokhan, S., Othman, R., Abd Rahim M.H., Karsani, S.A., & Yaacob, J.S. (2020). Effect of plant growth regulators on coloured callus formation and accumulation of azadirachtin, an essential biopesticide in *Azadirachta indica*. *Plants, 9*(3), 352-369. https://doi.org/10.3390/plants9030352.

Ashokhan, S., Othman, R., Abd Rahim M.H., Karsani, S.A., & Yaacob, J.S. (2019). Analysis of bioactive pigments in coloured callus of *Azadirachta indica* for possible use as functional natural colourants. *Pigment & Resin Technology, 48*(2), 9–19. https://doi.org/10.1108/PRT-11-2017-0095.

Bano, A., Khattak, M., Basit, A., Alam, M., Shah, S., Ahmad, N., Gilani, S., Ullah, I., Anwar, S., & Mohamed, H. (2022). Callus induction, proliferation, enhanced secondary metabolites production and antioxidants activity of *Salvia moorcroftiana* L. as influenced by combinations of auxin, cytokinin and melatonin. *Brazilian Archives of Biology and Technology*, *65*, 1-16. https://doi.org/10.1590/1678-4324-2022210200.

Bernabe-Antonio, A., Maldonado-Magana, A., Ramírez-Lopez, C.B., Salcedo-Perez, E., Meza-Contreras, J.C., Gonzalez-García, Y., Lopez-Dellamary Toral, F.A., & Cruz-Sosa, F. (2017). Establishment of callus and cell suspension cultures of *Eysenhardtia polystachya* (Ortega) and fungistatic activity of their extracts. *South African Journal of Botany, 112*, 40–47. https://doi.org/10.1016/j.sajb.2017.05.023.

Cabanas-Garcíam, E., Areche, C., Gomez-Aguirre, Y. A., Borquez, J., Munoz, R., Cruz-Sosa, F., & Perez-Molphe Balch, E. (2021). Biomass production and secondary metabolite identification in callus cultures of *Coryphantha macromeris* (Engelm.) Britton & Rose (Cactaceae), a traditional medicinal plant. *South African Journal of Botany*, *137*, 1-9. https://doi.org/10.1016/j.sajb.2020.10.002.

Chaâbani, G., Tabart, J., Kevers, C., Dommes, J., Khan, M., Zaoui, S., Chebchoub, L., Lachaâl, M., & Karray-Bouraoui, N. (2015). Effects of 2,4-dichlorophenoxyacetic acid combined to 6-Benzylaminopurine on callus induction, total phenolic and ascorbic acid production, and antioxidant activities in leaf tissue cultures of *Crataegus azarolus* L. var. aronia. *Acta Physiologiae Plantarum, 37*(2), 16. https://doi.org/10.1007/s11738-014-1769-4.

Daffalla, H. M., Elsheikh, A. M., Ali, H. A., & Khalafalla, M. M. (2019). Callus maintenance and cell line selection of *Grewia tenax*. *Journal of Herbs, Spices & Medicinal Plants*, *25*(3), 218-235. https://doi.org/10.1080/10496475.2019.1595256.

Daly, N. L. & Craik, D. J. (2011). Bioactive cystine knot proteins. *Current Opinion in Chemical Biology 15*(3), 362-368. https://doi.org/10.1016/j.cbpa.2011.02.008.

Dawa, K.K., El-denary, M.E., & Abo-elglagel, I.M. (2017). Callus formation and shoot regeneration as affected by plant growth regulators and explant types in three strawberry cultivars (*Fragaria x ananassa* Duch.). *Journal of Plant Production*, *8*(5), 599-604. https://doi.org/10.21608/JPP.2017.40481.

Dias, M.I., Sousa, M.J., Alves, R.C., & Ferreira, I.C.F.R. (2016). Exploring plant tissue culture to improve the production of phenolic compounds: a review. Industrial Crops and Products, *82*, 9–22. https://doi.org/10.1016/j.indcrop.2015.12.016.

Ebrahimzadeh, M. A., Navabi, S. M., Navabi, S. F., & Dehpour, A. A. (2011). Antioxidant activity of hydroalcholic extract of *Ferula gummosa* Boiss roots. *European Review for Medical and Pharmacological Sciences, 15*(6), 658-664.

El-Seedi, H. R., De Smet, P. A. G. M., Beck, O., Possnert, G., & Bruhn, J. G. J. (2005). Prehistoric peyote use: Alkaloid analysis and radiocarbon dating of archaeological specimens of *Lophophora* from Texas. *Journal of Ethnopharmacology*, *101*, 238-242. https://doi.org/10.1016/j.jep.2005.04.022.

Fiedler, I., Santacruz-Ruvalcaba, F., & Portillo, L. (2022). Establishment of callus and cell suspension cultures of *Ariocarpus retusus* (Scheidweiler). *Journal of the Professional Association for Cactus Development, 24*, 1-18. https://doi.org/10.56890/jpacd.v24i.454.

Fu, Z., Xu, P., He, S., Teixeira da Silva, JA., & Tanaka, M. (2011). Dynamic changes in enzyme activities and phenolic content during in vitro rooting of tree peony (*Paeonia suffruticosa* Andr) plantlets. *Maejo International Journal of Science and Technology*, *5*(2), 252–265.

Gonzalez-diaz, M.C., Perez-reyes, M.E., & Perez-molphe-blach, E. (2006). In vitro analysis of susceptibility to *Agrobacterium rhizogenes* in 65 species of Mexican cacti. *Biologia Plantarun*, *50*(3), 331-337. https://doi.org/10.1007/s10535-006-0077-2.

Gourguillon, L., Rustenholz, C., Lobstein, A., & Gondet, L. (2018). Callus induction and establishment of cell suspension cultures of the halophyte *Armeria maritima* (Mill.) Willd. *Scientia Horticulturae*, *233*, 407–411. https://doi.org/10.1016/j.scienta.2017.08.001.

Huang. G., Jin. Y., Zheng. J., Kang. W., Hu. H., Liu. Y., & Zou. T. (2016). Accumulation and distribution of copper in castor bean (*Ricinus communis* L.) callus cultures: in vitro. *Plant Cell Tiss Organ Cult*, *128*, 177-186. https://doi.org/10.1007/s11240-016-1097-z.

Huang, L.C., Lee, Y.L., Huang, B.L., Kuo, C.I., & Shaw, J.F. (2002) High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture. *In Vitro Cellular & Developmental Biology-Plant, 38*, 358–365. https://doi.org/10.1079/IVP2002298.

Hemmati, N., Cheniany, M., & Ganjeali, A. (2020). Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in *Salvia tebesana Bunge*. *Botanica Serbica*, *44*(2), 163-173. https://doi.org/10.2298/BOTSERB2002163H.

Hesami, M., Tohidfar, M., Alizadeh, M., & Daneshvar, M.H. (2018). Effects of sodium nitroprusside on callus browning of *Ficus religiosa*: an important medicinal plant. *Journal of Forestry Research*, *31*, 789–796. https://doi.org/10.1007/s11676-018-0860-x.

Huh, Y.S., Lee, J.K., & Nam, S.Y. (2017). Effect of plant growth regulators and antioxidants on in vitro plant regeneration and callus induction from leaf explants of purple passion fruit (*Passiflora edulis* Sims). *Journal of Plant Biotechnology*, *44* (3), 335-342. https://doi.org/10.5010/JPB.2017.44.3.335.

Hussain, M.S., Fareed, S., Ansari, S., Rahman, M.A., Ahmad, I.Z., & Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. *Journal of Pharmacy and Bioallied Sciences, 4*, 10–20. https://doi.org/10.4103/0975-7406.92725.

Jedinak, A., Faragó, J., Pšenáková, I., & Maliar, T. (2004). Approaches to flavonoid production in plant tissue cultures. *Biologia Bratislava*, *59*(6),697-710.

Kaewubon, P., Hutadilok-Towatana, N., &Teixeira da Silva, J.A. (2015). Ultrastructural and biochemical alterations during browning of pigeon orchid (*Dendrobium crumenatum* Swartz) callus. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *121*, 53–69. https://doi.org/10.1007/s11240-014-0678-y.

Kinoshita, K., Takizawa, T., Koyama, K., Takahashi, K., Kond,o N., & Yuasa, H. (1995). New triterpenes from *Trichocereus pachanoi. Journal of Natural Products, 58*(11), 1739–1744. https://doi.org/10.1021/np50125a016.

Llamoca-Zárate, R.M., Studart-Guimar~aes, C., Landsmann, J., & Campos, F.A.P. (1999). Establishment of callus and cell suspension cultures of *Opuntia ficus-indica*. *Plant Cell, Tissue and Organ Culture*, *58*, 155–157. https://doi.org/10.1023/A:1006315729266.

Luczkiewicz, M., Kokotkiewicz. A., & Glod, D. (2014). Plant growth regulators affect biosynthesis and accumulation profile of isoflavone phytoestrogens in high-productive in vitro cultures of *Genista tinctoria*. *Plant Cell, Tissue and Organ Culture*, *118*, 419–429. https://doi.org/10.1007/s11240-014-0494-4.

Mashayekhi, K., & Atashi, S. (2018). *Guidance of Plant physiology tests (pre- and post-harvest examinations of plants).* Gorgan: Agricultural Education Research Publications.

Martinez-Valverde, I., Periago, M.J., & Ros, G. (2000). Nutritional importance of phenolic compounds in the diet. *Archivos Latinoamericanos de Nutricion*, *50*(1), 5–18.

Medeiros, L.A., Ribeiro, R., Gallo, L.A., Oliveira, E.T., & Demattè, M. (2006). Invitro propagation of Notocactus magnificus. *Plant Cell, Tissue and Organ Culture, 84,* 165-169. https://doi.org/10.1007/s11240-005-9014-x

Mengesha, B., Mekbib, F., & Abraha, E. (2016). In vitro screening of cactus (*Opuntia ficus-indicia* (L.) Mill) genotypes for drought tolerance. *American Journal of Plant Sciences, 7*, 1741-1758. https://doi.org/10.4236/ajps.2016.713163.

Mondragón-Jacobo, C., & Chessa, I. (2013). A global perspective on genetic resources of cactus pear; an asset for the future sustainability of semiarid lands. *Acta Horticulturae, 995*, 19-26. https://doi.org/10.17660/ActaHortic.2013.995.1

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum, 15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nishchal, N., Mir, H., Rani, R., & Pal, A. K. (2018). Effect of antioxidants in controlling phenol exudation in micropropagation of Litchi cv. Purbi. *Current Journal of Applied Science and Technology, 31*(4), 1-7. https://doi.org/10.1590/2447-536x.v27i1.2230

Panche, A.N., Diwan, A.D., & Chandra, S.R. (2016). Flavonoids: an overview. *Journal of Nutritional Science*, *5*, e47. https://doi.org/10.1017/jns.2016.41.

Pádua, M., Paiva, L., Silva, L., Livramento, K., Alves, E., & Castro, A. (2014). Morphological characteristics and cell viability of coffee plants calli. *Ciência Rural*, *44*(4),660-665. https://doi.org/10.1590/S0103-84782014000400014.

Raj, P., Jakhar, M.L., Ahmad, S., Chahar, S., Kassim, Y. M., & Jat, H.R. (2020). A study on effects of antioxidants in micropropagation of Bael (*Aegle marmelos L.*). *Journal of Pharmacognosy and Phytochemistry, 9*(1), 1687-1690.

Rameshi, H. (2015). Bioreactors and optimal use of ecological potential of medicinal plants and plant secondary metabolites. *The 10th Congress of Pioneers of Progress*, 3292-3298. SID. https://sid.ir/paper/866083/fa

Reyes-Martínez, A., Antunes-Ricardo, M., Gutierrez-Uribe, J., & Santos-Díaz, M.d.S. (2019). Enhanced production and identification of antioxidants in in vitro cultures of the cacti *Mammillaria candida* and *Turbinicarpus laui*. *Applied Microbiology and Biotechnology*, *103*, 2583–2595. https://doi.org/10.1007/s00253-019-09656-8.

Robbins, R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry, 51*(10), 2866-2887. https://doi.org/10.1021/jf026182t

Robles-Martıinez, M., Barba-de la Rosa, A. P., Gueraud, F., Negre-Salvayre, A., Rossigno, M., & Socorro Santos-Diaz, m. D. (2016). Establishment of callus and cell suspensions of wild and domesticated *Opuntia* species: study on their potential as a source of metabolite production. *Plant Cell, Tissue and Organ Culture*, *124*, 181–189. https://doi.org/10.1007/s11240-015-0886-0.

Salmalian, M. (2017). *Studying the role of growth stimulants in biomass and changes in the biochemical composition of stevia (Stevia rebaudiana* Bertoni*) callus in solid and liquid culture conditions*. Gorgan: Gorgan University of Agricultural Sciences and Natural Resources.

Singleton, V. L. & Rossi, J. R. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungestic acid reagent. *American Journal of Enology and Viticulture*, *16* (3), 144-158.

Stahlschmidt, Z.R., DeNardo, D.F., Holland, J.N., Kotler, B.P., & Kruse-Peeples, M. (2011). Tolerance mechanisms in North American deserts: biological and societal approaches to climate change. *Journal of Arid Environments, 75*, 681–687. https://doi.org/10.1016/j.jaridenv.2011.03.006.

Sun, L., Zhang, J., Lu, X., Zhang, L., & Zhang, Y. (2011). Evaluation to the antioxidant activity of total flavonoids extract from persimmon (*Diospyros kaki* L.) leaves. *Food and Chemical Toxicology*, *49*(10), 2689-2696. https://doi.org/10.1016/j.fct.2011.07.042.

Taghizadeh, M., & Dastjerdi, M. G. (2020). Inhibition of browning problem during the callogenesis of *Spartium junceum* L. *Ornamental Horticulture, 27*, 68-77. https://doi.org/10.1590/2447-536X.v27i1.2230

Tan, S. H., Musa, R., Ariff, A., & Maziah, M. (2010). Effect of plant growth regulators on callus, cell suspension and cell line selection for flavonoid production from pegaga (*Centella asiatica* L. urban). *American Journal of Biochemistry and Biotechnology, 6*, 284–299. https://doi.org/10.3844/ajbbsp.2010.284.299.

Tang, W. & Newton, R.J. (2004) Increase of polyphenol oxidase and decrease of polyamines correlate with tissue browning in Virginia pine (*Pinus virginiana Mill.*). *Plant Science, 167*, 621–628. https://doi.org/10.1016/j.plantsci.2004.05.024.

Teodora, V. I., Nicoleta, L. A., & Stefania, S. A. (2015). Study on the regenerative capacity and organogenic of *Echinopsis* (Zucc.) *chamaecereus f. Lutea* explants, in the presence of 2,4-Dichlorophenoxyacetic acid (2,4-D) in culture medium. *Analele Universitaţii din Oradea, Fascicula Protecţia Mediului*, *25*, 133-140.

Vidican, T. I., Cachita-Cosma, D., & Romocea, J. E. (2009). The initiation of *Echinocactus mihanovichii*, *Echinopsis chamaecereus, f. lutea* and *Aylostera heliosa* vitrocultures. *Studia Universitatis Vasile Goldis, Seria Stiintele Vietii, 19*(2), 351-357.

Wang, J., Li, J.-L., Li, J., Li, J.-X., Liu, S.-J., Huang, L.-Q., & Gao, W.-Y. (2017). Production of active compounds in medicinal plants: from plant tissue culture to biosynthesis. *Chinese Herbal Medicines, 9*, 115–125. https://doi.org/10.1016/S1674-6384(17)60085-6.

Wickremesinhe, E. R., & Arteea, R. N. (1993). Taxus callus cultures: initiation, growth optimization, characterization and taxol production. *Plant Cell, Tissue and Organ Culture, 35*, 181-193. https://doi.org/10.1007/BF00032968

Wongsen, W., Bodhipadma, K., Noichinda, S., & Leung, D. W. M. (2015). Influence of different 2,4-D concentrations on antioxidant contents and activities in sweet basil leaf-derived callus during proliferation. *International Food Research Journal*, *22*(2), 638-643.

Young Choa, H., Young Sona, S., Soon Rheea, H., Sung-Yong, H., Yoonb Carolyn, W. T., Parsonsc Jong, L., & Parka M. (2008). Synergistic effects of sequential treatment with methyl jasmonate, salicylic acid and yeast extract on benzophenanthridine alkaloid accumulation and protein expression in *Eschscholtzia californica* suspension cultures. *Journal of Biotechnology*, *135*(1), 117–122. https://doi.org/10.1016/j.jbiotec.2008.02.020.

Zhou, F., Wang, Z., Shi, L., Niu, J., Shang, W., He, D., & He, S. (2016). Effects of different medium composition and exogenous hormones on browning of tree peony (*Paeonia suffruticosa* Andr.) callus in tissue culture. *Flower Research Journal*, *24*, 96-102. https://doi.org/10.11623/frj.2016.24.2.03.