Plant Growth Regulators Promoting Callus Induction and Antioxidant Activity in White Sapote (*Casimiroa Edulis*)

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ABSTRACT

White sapote is a fruit that has many biological effects on human health including antidiabetic, antidepressant, antioxidant, anticancer and immunomodulatory activities. It contains various dietary constituents including phenolics, carotenoids, polysaccharides and vitamins. The in vitro culture system should provide the best way to develop and produce the valuable biological compounds of White sapote. To establish a protocol for callus induction of White sapote and compare its antioxidant activity in callus culture from different explants, plant growth regulators (also known as PGRs) were applied in several treatments. Various explants were grown on MS media. These media contained PGRs in five different treatments, including a combination of Benzyl Adenine (BA) with Naphthalene Acetic Acid (NAA). Most of the evaluated explant types produced 100% callus induction. (nodal segments, shoot tips, and root segments) on a medium provided 0.5 mg/L BA with 2 mg/L NAA; however, leaf segments didn't produce any callus. Under the medium including 2 mg/L NAA along with 0.5 mg/L BA, shoot-tip explants produced the largest callus fresh mass (1.05 gm). The amounts of antioxidant activity were estimated and the noticeable difference was acquired from various PGR treatments in the callus. These findings suggest that specific PGRs increase the accumulation of different secondary metabolism products and have influence on the production of callus from white sapote. Thus, the culture protocol detailed in this study may provide a novel method for micropropagation of this plant from callus and for secondary metabolic products from white sapote.

Keywords: callus induction; plant growth regulators; antioxidant enzymes; proline.

INTRODUCTION

White sapote (*Casimiora edulis*) is a fruit tree that is native to Mexico and Central America (Morton, 1987). It is grown both as a cultivated and wild species in central and southern Mexico, as well as in Guatemala, El Salvador, and Costa Rica (Hailu and Wakgari, 2019). White sapote is a fruit that thrives in subtropical climates and produces large and sweet fruit (Yamamoto *et al*., 2007). Due to its therapeutic properties, it is extensively grown in home gardens and traded commercially in Florida and California in the United States, as well as in Australia, New Zealand, and Japan (Yonemoto *et al*., 2007). The tree is also grown on a small scale in South Africa, Egypt, and different parts of Ethiopia (Zegeye *et al*., 2011; Abo Taleb and Abdul Latif, 2023). In Egypt, it is cultivated for its edible fruit, and its medicinal properties are also recognized (Khalil *et al*., 2022). The demand for alternative medicines has increased significantly, therefore leading to a rise in the use of phytotherapy.

This has resulted in more research towards finding new active principles. To achieve this, it is essential to establish *in vitro* callus culture model that can produce relevant substances with pharmacological properties (López Arnaldos *et al*., 2001; Lima *et al*., 2007; Morais *et al*., 2012). Callus culture systems have proven to be a reliable means of producing significant amounts of secondary compounds. In fact, the levels of production in callus cultures often exceed those achieved by intact plants. This method of production has the added benefit of eliminating the problems associated with traditional cultivation, e.g., seasonality and the need for pesticides. Furthermore, production can be increased by breeding approaches and by optimizing the *in vitro* conditions (Oliveira *et al*., 2009; Morais *et al*., 2012; Elshahawy *et al*., 2022).

This study aimed to develop a protocol for callus induction for the indirect vegetative propagation process and for the synthesis of secondary substances *in vitro* from White sapote (*C. edulis*)

MATERIALS AND METHODs

This research was conducted at the Plant Physiology laboratory in the Department of Agricultural Botany, Faculty of Agriculture at Cairo, Al-Azhar University, Egypt. The research aimed to identify the most efficient treatments for the development of white sapote (*C. edulis*) callus induction and compare

antioxidant activity in callus from different explants.

Plant material

Shoot tips, leaf, nodal and root segments, were obtained from 3-month-old white sapote healthy seedlings grown *in vitro* (Fig.1).

***In vitro* germination**

White sapote fruits were collected from a healthy and fruitful tree on the farm of the Horticulture Department at the Faculty of Agriculture, Al-Azhar University, Cairo, Egypt. After the seeds were removed from the fruits, they were surface sterilized for five minutes with hydrochloric acid. Following that, seeds were washed with distilled and sterilized water for 5-6 minutes, 3-4 times. The coat of the seeds was removed (Muralidhara *et al.*, 2023) before being inoculation onto jars that contained hormone-free MS basal medium. All processes were performed in a laminar airflow chamber.

Preparation of medium

The explants tested were cultivated on Murashige and Skoog (1962) (MS) basal medium enriched with 3% sucrose and supplemented with 250 mg\L Cefotaxime (Ateto *et al.*, 2018). Different combinations and concentrations of NAA as auxin and BA as cytokinin to the medium at the following concentrations

1.0 mg/l NAA + 0.25 mg/L BA

2.0 mg/l NAA + 0.25 mg/l BA

2.0 mg/l NAA + 0.50 mg/L BA

2.0 mg/l NAA + 1.0 mg/L BA

2.0 mg/l NAA + 2.0 mg/L BA

The pH of the media was set to 5.8 and agar (0.8%) was used as an additive for solidification. Sucrose (3%) was added as a carbohydrate source. MS basal medium was exploited as a negative control. The cultures were then incubated in a culture room, and the results were recorded after 4 weeks. The medium was autoclaved after dispensing into appropriate culture vessels.

Culture conditions

The cultures were incubated in a plant growth chamber maintained at 23±5°C with 55-65% relative humidity and exposed to a 16/8 hours light/darkness regime of 2500-3000 lux intensity supplied via white fluorescent tubes.

Determination of proline content

Proline was extracted from 200 mg of fresh tissue samples by homogenizing in 4 ml of 3% aqueous sulfosalicylic acid. The extraction method followed what is described in Bates *et al.* (1973). Briefly, centrifugation at 10000 rpm was performed after which 2ml of the supernatant was mixed alongside 2 ml of glacial acetic acid and 2 ml of ninhydrin and then kept for 1 hour at 100°C. After separating the combination with four ml of toluene, the absorbance of the resulting substance was determined using a spectrophotometer at 520 nm. A standard curve was used to determine the proline amounts.

Antioxidant enzymes determination

The study evaluated the activity of three antioxidant enzymes, namely polyphenol oxidase (PPO), peroxidase (POD), and catalase (CAT), using a spectrophotometer. The fresh tissues, weighing 200 mg, were ground in liquid nitrogen and homogenized to obtain a 4 mL solution containing 50 mM potassium phosphate buffer and 1% (w/v) PVP with a pH of 7. The homogenization was performed on an ice bath, followed by a 10-minute cold centrifugation at 14,000 rpm of the homogenate. The resulting supernatant was used for enzyme evaluations. The PPO activity was assessed using Duckworth and Coleman (1970) approach, while the POD activity was estimated using Chance and Maehly (1955) assay procedure. The catalase activity was determined using the technique outlined by Aebi (1984).

Statistical analysis

Student t-test was employed to compare the means between the different treatments.

RESULTS AND DISCUSSIONS

The impacts of various explants and diverse treatments from PGRs on callus formation were analyzed and are shown in Tables 1 and 2 and Figures 2 and 3. The different amounts of auxins and cytokinins, as well as the explant sources, caused noticeable variances in the callus induction percentage, callus fresh weight, and callus dry weight. The results demonstrated that callus varied based on PGR treatments. Below is a description of how PGRs and the explant's origin affect callus production.

Effect of interaction between NAA, BA and explant type on callus induction

Table (1) shows data on the induction rate of callus under five treatments of two PGR, BA and NAA. The results indicate that the

induction rate is influenced by the concentration levels of these regulators. Additionally, the trend of callus induction rate demonstrates an initial increase, followed by a decrease with increasing BA concentration. This trend is slightly similar to what is found by Assem *et al.* (2014) on the adverse effect of cytokinin on callus induction. Notably, the treatment using 2 mg/L NAA in addition 0.5 mg/L BA displayed best callus induction percentage, reaching an impressive 100%, a result similar to that of Martínez *et al.* (2006). All the used explants produced callus except for leaves, which did not respond to any of the tested treatments (data not shown), this finding also reported by (Karakas, 2020). The best-performing explant was root segments (90%), followed by nodal segments (71.43%) and shoot tips (71%). The diverse *in vitro* responses exhibited by each type of explant might be attributed to the disparities in epigenetic regulation, along with the presence of endogenous sugars and phytohormones (Duta-Cornescu *et al.*, 2023; Ai *et al.*, 2024; Jafari and Daneshvar, 2024).

Effect of interaction between NAA, BA and explant type on callus fresh and dry weight

Table (2) shows and indicates that the interaction between the concentrations of auxin and cytokinin was significant in affecting the callus's fresh weight. The explants treated with 2 mg/L NAA and 0.25 mg/L BA had the best mean fresh weight (804.92 mg), while the callus generated from the shoot tip treated with 2 mg/L NAA and 0.5 mg/L BA had the highest fresh weight (1055 mg). These results are consistent with what Prior research revealed that the existence of cytokinins and auxins in the nutritional medium has a role in inducing callus and increasing its growth, and encourages The processes of cell division and growth with an increase in the manufacture of basic contents to increase division and growth, such as amino acids and proteins, and lead to an increase in the biomass of the callus and then a rise in the fresh weight rates (Lu *et al.*, 2023; Mansour *et al.*, 2024).

Effect of interaction between NAA, BA and explant type on antioxidant activity

Proline content

In this study conducted in vitro, treatment with five different combinations of GPRs resulted in changes in proline content in callus formed in white sapote, ranging from 1.39 (µmol/g FW) to 18.55 (µmol/g FW) (Fig. 3). Interestingly, while application of 1 mg/L BA along with 2 mg/L NAA decreased the proline content, the other treatments increased it in callus cultures of white sapote (Fig. 3). The use of 0.5 mg/L BA alongside 2 mg/L NAA produced the most elevated proline levels in shoot tip and root segment callus, whereas in nodal segment callus, The maximum proline content was detected with a application of 1 mg/L NAA plus 0.25 mg/L BA.. According to Karakas and Bozat (2020), media composition affected proline content in callus.

Enzymatic antioxidant activities

The outcomes of the study demonstrated that the highest activity of the polyphenol oxidase enzyme (PPO) was noticed in the shoot tip callus when dealt with 2 mg/L NAA alongside 0.25 mg/L BA. On the other hand, the nodal-segment callus showed the most intense activity for this enzyme when media supplied with 2 mg/L NAA plus 0.5 mg/L BA, while the root-segment callus revealed the best result when dealing with 2 mg/L NAA in addition to 1 mg/L BA as shown in (Fig. 3). The peroxidase enzyme (POD) activity shows the highest activity in callus of all tested explants when treated with 1 mg/L NAA and 0.25 mg/L BA as shown in (Fig. 3). Lastly, the catalase enzyme (CAT) showed the highest activity when treated with 2 mg/L NAA and 0.25 mg/L BA in the shoot tip callus and root segment callus. However, in the nodal-segment callus, the highest activity was observed when media provided with 2 mg/L NAA together with 1 mg/L BA as shown in (Fig 3). These findings supporting Didi *et al.* (2022) conclusion that exogenous PGRs influence the activity of antioxidant enzymes.

CONCLUSION

Our data suggest that certain combinations of PGRs may improve the callus induction and the synthesis of secondary compounds in the callus cultures of *C. edulis*. NAA and BA are effective for callus induction in shoot tips, nodal segments and root segments explants of *C. edulis*, however, leaf segments didn't produce any callus. The application of 2.0 mg/1 NAA along with 0.5 mg/L BA results in high callus induction, with an average of 1.05 grams of fresh mass in 4 weeks of cultivation. The antioxidant activity varied in callus from different PGRs combinations, leading to varied accumulation of secondary metabolites and affecting white sapote callus production. These findings suggest that the choice of PGRs can impact the antioxidant activity and secondary metabolite production in white sapote callus, though further research is needed to fully understand the molecular mechanisms

underpinning the variations and to optimize callus production for potential applications in the nutritional and medicinal sectors.

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Table 1: Effect of NAA and BA on the callus induction using different explants of white sapote (*C. edulis*)

Treatments (mg/L)			Callus induction (%)			
No	NAA	BA	Shoot tip	Nodal segment	Root segment	Mean
1	1	0.25	75.00	100.00	100.00	91.67[a]
2	2	0.25	100.00	85.71	87.50	91.07[a]
3	2	0.50	100.00	100.00	100.00	100.00[a]
4	2	1.00	25.00	14.29	62.50	33.93[b]
5	2	2.00	50.00	57.14	100.00	69.05[a]
Mean			70.00	71.43	90.00	

Means with different letters differ significantly at P value = 0.05

Table 2: Effect of NAA and BA on the callus fresh weight (FW) and dry weight (DW) using different explants of white sapote (*C. edulis*)

Treatments (mg/L)			Shoot tips callus		Nodal segments callus		Root segments callus		Mean	
No	NAA	BA	FW (mg)	DW (mg)	FW (mg)	DW (mg)	FW (mg)	DW (mg)	FW (mg)	DW (mg)
1	1	0.25	330.00	32.20	259.25	23.75	702.60	34.60	430.62[a]	30.18[a]
2	2	0.25	936.50	39.80	645.00	36.38	833.25	30.88	804.92[a]	35.68[a]
3	2	0.50	1055.00	59.00	603.25	32.45	563.33	22.13	740.53[a]	37.86[a]
4	2	1.00	90.00	13.70	168.00	14.10	406.00	26.75	221.33[b]	18.18[a]
5	2	2.00	736.00	54.10	643.67	47.60	418.20	26.36	599.29[a]	42.69[a]
Mean			629.50	39.76	463.83	30.86	584.68	28.14		

Means with different letters differ significantly at P value = 0.05

Figure 1: White sapote 3-month-old seedlings grown ***in vitro*** used as a source of sterilized explants.

Treatments (mg/L)			**Explant type**		
No	**NAA**	**BA**	**Shoot tip**	**Nodal segment**	**Root segment**
1	1	0.25			
2	2	0.25			
3	2	0.50			
4	2	1.00			
5	2	2.00			

Figure 2: Different callus obtained using various combinations of NAA and BA from diverse explants of white sapote (*C. edulis*) after 4 weeks.

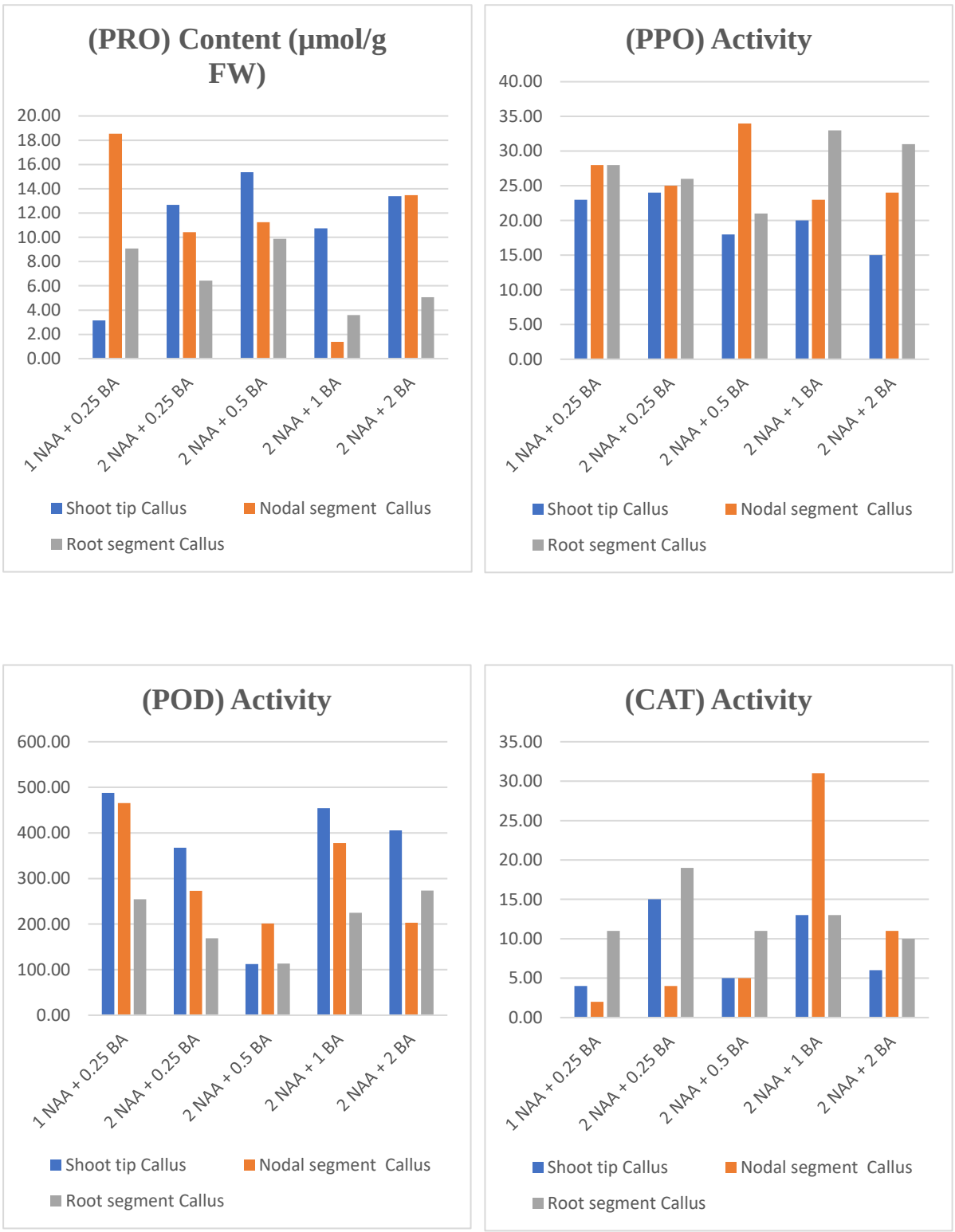

Figure 3: Effect of interaction between NAA, BA and explant type on antioxidant activity of *C. edulis*.

منظمات النمو النباتية المحفزة لاستحثاث الكالس ونشاط مضادات الأكسدة في السابوتا البيضاء

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الملخص العربي

السابوتا البيضاء هي فاكهة لها العديد من التأثيرات البيولوجية على صحة الإنسان مثل مضاد لمرض السكري، ومضاد للأكسدة، ومضاد للسرطان، ومضاد للاكتئاب، ومنظم للمناعة. ويحتوي على العديد من المكونات الغذائية مثل الفينولات والفيتامينات والكاروتينات والسكريات. ويمكن أن يوفر تكنيك زراعة الأنسجة أفضل طريقة لتطوير وإنتاج المركبات البيولوجية الهامة للسابوتا البيضاء. لإنشاء بروتوكول لاستحثاث الكالس من منفصلات نباتية مختلفة من نبات السابوتا البيضاء ومقارنة نشاط مضادات الأكسدة في مزارع الكالس من منفصلات نباتية مختلفة، تم تطبيق معاملات مختلفة من منظمات النمو النباتية. تم استخدام أنواع مختلفة من المنفصلات النباتية وزراعتها على وسط موراشيج وسكوج (MS) يحتوي على خمسة تركيزات مختلفة من منظمات النمو النباتية [نفثالين حمض الخليك (NAA) والبنزيل أدنين (BA)]. تم الحصول على أعلى متوسط لتكوين الكالس (100%) من المنفصلات النباتية (القمم النامية، القطع العقدية، والقطاعات الجذرية) على وسط يحتوي على 2 ملغم/لتر NAA و0.5 ملغم/لتر BA. أعلى وزن رطب للكالس (1.05 جم) من المنفصلات النباتية على وسط يحتوي على 2 ملغم/لتر NAA و0.5 ملغم/لتر BA. تم تقدير نشاط مضادات الأكسدة حيث أظهرت فروق كبيرة في الكالس الذي تم الحصول عليه من معاملات مختلفة من منظمات النمو النباتية. تشير هذه النتائج إلى أن منظمات النمو النباتية المختلفة تؤدي إلى تراكم المزيد من أنواع مختلفة من المستقلبات الثانوية وتؤثر على إنتاج الكالس في السابو البيضاء. يمكن استخدام بروتوكول زارعة الأنسجة الموضح في هذه الدراسة فى التكاثر الدقيق لهذا النبات أيضا كطريقة جديدة للإنتاج التجاري لنواتج الأيض الثانوية للسابوتا البيضاء على مستوى الكالس.

الكلمات الاسترشادية: استحثاث الكالس، منظمات النمو النباتية، مضادات الأكسدة الإنزيمية، البرولين.