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An Improved Protocol for Callus Initiation Using Leaf Discs and Stems as Explants from Yellow Passion Fruit (*Passiflora edulis f. flavicarpa)*

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ABSTRACT

Passiflora edulis f. *flavicarpa* is a perennial vine mainly grown in tropical countries which has agricultural and horticultural importance. Yet, the unavailability of an efficient micropropagation technique is a major obstacle to overcome. Different surface sterilization methods were tested for the stem and leaf explants. To determine the best plant growth regulator concentrations for callus induction, different combinations of NAA (2 to 5 mg/L) and BAP (0.1 to 0.3 mg/L) were tested. Murashige and Skoog full-strength medium was used as the basal medium for cultures. The use of 10% Clorox™ at 150 rpm for 12 minutes twice, followed by 0.1 g/L $AgNO_3$ for 12 minutes found to be the most effective surface sterilization method for leaves with a 100% survival rate. Results found 0.2 mg/L NAA; 2 mg/L BAP and 0.1 mg/L NAA; 4 mg/L BAP to be optimal for callus induction from leaves under light and dark conditions with calli induction rates of 33.33% and 16.67%, respectively. For stems best results were obtained for 0.3 mg/L NAA; and 2 mg/L BAP with a 66.67% calli induction rate in light conditions and 33.33% in dark conditions. Calli were obtained from both explants within an average of 38.5 days and 44 days in light and dark conditions respectively. Calli of yellow passion fruit can be obtained using full MS media supplemented with NAA ranging from 0.1 to 0.3 mg/L and BAP ranging from 2 to 4 mg/L. Despite successes, vitrification and contamination underscore the need for refinements on this protocol.

Keywords: Yellow passion fruit; callus; micropropagation; NAA; BAP

Introduction

Passiflora edulis f. *flavicarpa*, or yellow passion fruit, is a tropical perennial semi-woody vine which belongs to the *Passifloraceae* family and *Passiflora* genus indigenous to Brazil (Amugune, 1992; Silva & Souza, 2020; Taïwe & Kuete, 2017). Sri Lanka, being a tropical country stands as one of the largest producers of passion fruit in the world. Bandarawela purple, Rahangala hybrid, and Horana gold are the passion fruit varieties cultivated in Sri Lanka, while Horana gold stands as the most popular cultivar (Department of Agriculture Sri Lanka, 2023).

Horana gold is popular for its distinctively attractive flowers and acidic fruit. The fruit of the plant holds significance in producing juice, jam, tea, and ice cream. Due to the rich composition of bioactive compounds such as vitamin C, beta-carotene, and flavonoids passion fruit juice has high antioxidant content (Viuda-Martos et al., 2020; Wijeratnam, 2015). Research has shown that the consumption of yellow passion fruit helps reduce inflammation, alleviate anxiety symptoms, regulate blood sugar

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levels, and protect against oxidative stress-related diseases such as certain types of cancer (Rai et al., 2022).

The primary methods employed to propagate yellow passion fruit are stem cuttings and seed propagation (Taïwe & Kuete, 2017). Even though, stem cuttings are preferred as they ensure that the produced plants are visually identical to the mother plants, due to microbial attacks stem cuttings are not considered to be the best option and cross-pollination makes seeds also not to be the best propagation method.

As Sri Lanka is one of the world's largest producers of *Passiflora*, applying micropropagation techniques to advance the market will be of great use. Therefore, it is essential to develop an enhanced and standardized micropropagation protocol specific to yellow passion fruit. Even though, micropropagation techniques have been widely studied, the unique requirements and challenges posed by *P. edulis* f. *flavicarpa* remain relatively unexplored. Though previous studies in different countries have examined the use of leaf disc explants in the micropropagation of different varieties of passion fruit, there is a notable inconsistency and diversity in the reported timeframes required for callus formation specifically on the yellow passion fruit variety (Mukasa et al., 2016; Ozarowski & Thiem, 2013; Senanayake & Silva, 2023; Tuhaise et al., 2019).

This research was undertaken with the comprehensive consideration of various factors to figure out optimal combinations of cytokinin and auxin for callus induction using leaf discs and stems as explants.

Materials and Methods

The research was conducted in the plant tissue culture laboratory at the Sri Lanka Institute of Information Technology, Malabe, Sri Lanka. Explants were obtained from domestically cultivated healthy plants of Horana gold*,* in Kaduwela and Kandy, Sri Lanka.

Explants were individually cleaned using Vim™ and subsequent experiments involving various surface sterilization protocols were performed using different concentrations and combinations of Isopropyl alcohol (IPA), Clorox™, Fungicide and Silver nitrate ($AgNO_3$) as shown in Table 1 for leaf discs. The protocol described by Kodithuwakku and Silva (2020) was followed for stem explants.

For the sterilization of leaves:

Surface sterilization was carried out as mentioned in Table 1 and was followed by 5.0 mL/L Fungicide for 10 minutes in methods 7,8 and 9, and 0.1 g/L AgNO3 for 10 and 12 minutes in methods 10 and 11 respectively.

Table 1. The summary of surface sterilization methods for leaf explant

Method	Isopropyl Alcohol		Clorox™		
	Amount (%)	Time (Min.)	Amount (%)	Time (Min.)	RPM
1	50	5	3	5	50
2	50	5	4	5	50
3	50	5	5	5	50
4	70	5	6	5	50
5	70	5	8	5	50
6	70	5	10	5	50
7	NA	NA	8 (twice)	12	125
8	NA	NA	10 (twice)	12	125
9	NA	NA	12 (twice)	12	125
10	NA	NA	10 (twice)	12	180
11	NA	NA	10 (twice)	12	150

Establishment of explants in culture tubes

All glassware and plasticware were sterilized using the CSUP technique (Peiris et al., 2012). The basal medium for culturing was the Murashige and Skoog (MS) medium supplemented with 3% sugar and myoinositol (100mg/L) (Murashige & Skoog, 1962). Various plant growth regulator combinations were tested to determine the best plant growth regulator combination and concentration as shown in Table 2.

Table 2. Summary of the plant growth regulator combinations used for the callus induction

Media Number	Used Explant	Plant growth regulator concentrations	
		mg/L	BAP (mg/L)
1	Leaf	0.1 NAA	2.0
2	Leaf	0.1 NAA	3.0
3	Leaf	0.2 NAA	4.0
4	Leaf	3.0 - 2,4-D	2.0
5	Leaf	2.0 - 2,4-D + 0.8 IAA + 1.0 KIN	2.0
6	Stem	None	4.0
7	Stem	None	3.0
8	Leaf	0.2 NAA	3.0
9	Leaf	0.3 NAA	2.0
10	Leaf	0.3 NAA	3.0
11	Leaf	0.1 NAA	4.0
12	Leaf	0.2 NAA	2.0
13	Leaf	0.2 NAA	4.0
14	Leaf/ Stem	0.2 NAA	5.0
15	Leaf	0.3 NAA	4.0
16	Stem	None	4.0
17	Leaf	0.3 NAA	5.0
18	Leaf/ Stem	0.1 NAA	5.0
19	Stem	None	5.0
20	Leaf/ Stem	0.2 NAA	3.0
21	Leaf/ Stem	0.3 NAA	2.0
22	Leaf	None	None
23	Leaf	3.0 - 2,4-D	2.0
24	Leaf	0.2 NAA	3.0
25	Leaf	0.3 NAA	2.0

Leaf explants were sectioned into 1 cm^2 squares and stems were cut into 1 cm segments and placed in culture tubes. They were exposed to light and dark conditions. The cultures were initially monitored for 72 hours and then regularly observed afterwards for any changes. Any cultures found to be contaminated or showing signs of vitrification were promptly discarded from the storage area (temperature – 25 C ± 2; Humidity - 40% 60%).

Data collection and analysis

The survival rates for each surface sterilization method were calculated using the following formula.

$$Mean\ Survival\ Rate = \frac{Number\ of\ survived\ cultures\ (72h)}{Total\ Number\ of\ Cultures} \times 100$$

The dataset was analysed utilizing Microsoft Excel software. A one-way analysis of variance (ANOVA) was performed with significance levels established at $P < 0.05$ considering 25 replicates for each condition.

Results and Discussion

Optimization of surface sterilization

As Figure 1, depicts, though the first 4 treatments resulted in a 0% survival rate, the results progressively improved. By employing the surface sterilization method 8 (10% Clorox at 125 rpm for 12 minutes twice, followed by 5 mL/L fungicide for 10 minutes) a 100% survival rate was observed under the dark condition. A 100% survival rate under both light and dark conditions was achieved for method 10 (10% Clorox for 12 minutes at 180 rpm followed by 0.1 g/L AgNO3 for 10 minutes) and 11 (10% Clorox at 150 rpm for 12 minutes twice, followed by 0.1 g/L AgNO3 for 12 minutes). The rotation speed was reduced from 180 to 150 as the higher speed was found to damage the tissues of the explants. The efficacy of $AgNO_3$ in mitigating contamination within cultures noted by Bleecker & Kende (2000) and Kumar et al. (2009) was confirmed by this study. Consistent with this knowledge, this study also found the best surface sterilization technique for leaf explants with the treatment including $AgNO_3$.

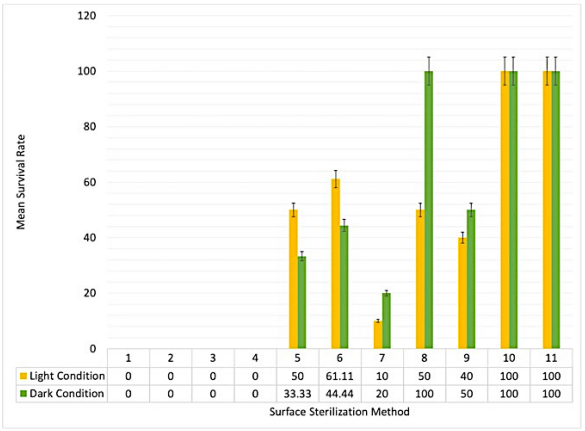

Figure 1. Survival Rate of cultures In Light and Dark Conditions for leaf and stem

Upon conducting an ANOVA to assess the relationship between survival rates and surface sterilization methods under light and dark conditions, a significant result with a P-value of 0.027 and 0.017 was obtained, respectively. As these p values are smaller than 0.05, a strong association was indicated between the survival rates and surface sterilization methods employed in the study.

Callus Induction

In media 11 (0.1 NAA; 4.0 BAP), 21 (0.3 NAA; 2.0 BAP) and 23 (23 3.0 2,4-D; 2.0 BAP) calli were observed in both lighting conditions. In leaf explant, calli formation was observed in media numbers 12 (0.2 NAA; 2.0 BAP), 23, and 8 (0.2 NAA; 3.0 BAP) (light conditions) and 11, 23, and 21 (dark conditions) and media 21 facilitated callus formation in both lighting conditions in stem explants. The observations suggest that the low auxin (NAA; 2,4-D) concentrations combined with a high concentration of cytokinin (BAP) were inducing calli formation in yellow passion fruit. In previous studies done on other passion fruit varieties, similar results have been obtained as reported by Ozarowski and Thiem in 2013.

Figure 2. shows the observed calli in the research. Observed calli was whitish in colour and originated from the explant margins in both stems and leaves.

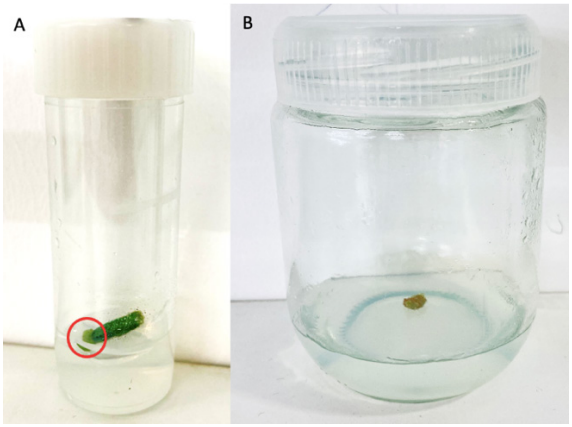

Figure 2. Observed calli in cultures (A – Observed calli in stem explant (44 days) | B – Isolated calli (90 days) 20th Media (0.2 NAA; 3.0 BAP) in dark condition

As shown in Figure 3, the highest incidence of callus formation from leaf explants was observed for media 12 (0.2 NAA; 2.0 BAP) and media 11 (0.1 NAA; 4.0 BAP) in light and dark conditions respectively. A 100% vitrified percentage was observed with media 11. The possible reason for this might have been the ratio of auxin to cytokine in this media. As noted by Lind and Rijkenberg in their paper that high concentrations of cytokine combined with a low auxin level can lead to vitrification because the cytokine can disrupt the normal cellular organization and water regulation.

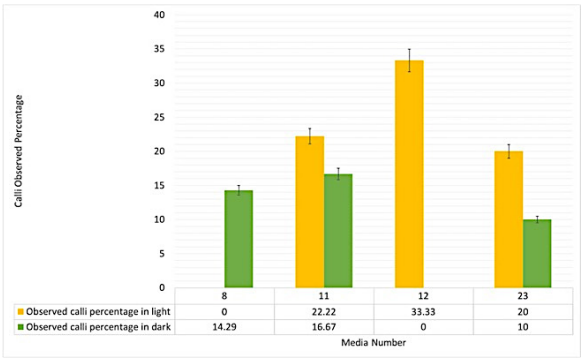

Figure 3. Percentage of calli observed in leaf explants

In stem explant, media 21 (0.3 NAA; 2.0 BAP) exhibited the highest calli observed percentage of 66.67% light conditions. Conversely, under dark conditions, media 20 (0.2 NAA; 3.0 BAP) and 21 demonstrated an equal highest percentage of 33.33%. These findings underscore the influence of both media composition and lighting conditions on the in-vitro responses of plant tissues, highlighting the complexity of their interactions in tissue culture systems (Ikeuchi et al., 2013). Furthermore, there were no observations of vitrified calli in dark conditions.

P values greater than 0.5 were obtained for callus induction rates in different media combinations. Vitrification and contaminations incite the need for further investigation into alternative factors impacting callus formation in yellow passion fruit tissue culture and refinements in the protocol.

Conclusions

The investigation identified the optimal surface sterilization technique for *P. edulis* f. *flavicarpa* leaf explant. Integrating $AgNO_3$ into sterilization protocols improved survival rates and reduced contamination

risks. The cultivation of calli in *P. edulis* f. *flavicarpa* is facilitated by the application of MS media supplemented with NAA ranging from 0.1 to 0.3 mg/L and BAP ranging from 2 to 4 mg/L. Despite successes, challenges such as vitrification and contamination underscore the need for ongoing protocol refinement to advance to be used in agriculture and genetic engineering.

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