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Enhancement of callogenesis from plumular explants of coconut *(Cocos nucifera)* via exogenous supplementation of amino acids and casein hydrolysate

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ABSTRACT

The role of exogenous supplementation of amino acids and casein hydrolysate in enhancing callogenesis from plumular explants of coconut (*Cocos nucifera* L.) was studied at ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala, during 2020-21. The combination of three amino acids, *viz.* glutamine, asparagine, proline, and organic additive casein hydrolysate were supplemented in three types of basal media (Eeuwen's Y3, Murashige and Skoog (MS) and 1/2MS) containing 2, 4-D (16.5 mg/L), charcoal (0.1%) sucrose (3%) and agar (0.8%). A total of 19 treatments were formulated and tested for production of embryogenic colloid tissue. The explants in glutamine-supplemented medium generated better colloid tissue and showed positive effect on colloid tissue multiplication, compared to other combinations. Of three basal media, Y3 was better over 1/2MS and MS basal media. These results could be useful for improving the efficiency of *in-vitro* regeneration in coconut by somatic embryogenesis from plumular explants.

Key Words: Embryogenic calli, Amino acids, Basal media, Plumule, Callogenesis, Explants

Coconut (*Cocos nucifera* L., 2n=32) is propagated via seed/nut. Only a limited number of seedlings can be produced from a single mother palm and its uniformity is not guaranteed due to its cross-pollinating nature. Coconut is most. Improving the formation of callus or colloid-like tissue from the explant and multiplication of these are of major interest in recalcitrant palms like coconut for improving the existing clonal propagation protocol of coconut via somatic embryogenesis from plumular tissues. Exogenously added amino acids and additional organic supplements play a vital role in plant micropropagation. Culture mediums are rarely supplemented with additional amino acids and organic supplements. Use of amino acids enhanced the somatic embryogenesis and *in-vitro* regeneration potential in several monocots' species. Therefore, three amino acids and one organic supplement were tested along with three basal media to test the effect of these additional components on callogenesis from plumular explant of coconut.

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MATERIALS AND METHODS

Mature nuts of 11-12 months old nuts were harvested from West Coast Tall (WCT) palms. Embryos with endosperm were excised from split nuts using a cork borer and were washed thoroughly using tap water followed by one wash with distilled water. Washed endosperm plugs enclosing the embryos were subjected to sterilization with 0.01% $HgCl_2$ for 3 minutes and washed again with sterile distilled water to remove the traces of heavy metal. Subsequently, embryos were taken out carefully from endosperm plug using a surgical blade under laminar airflow chamber. Extracted embryos were further treated with 20% NaClO for 20 minutes under laminar airflow chamber, after decanting the NaClO, embryos were rinsed using sterile distilled water 5-6 times to remove the traces of NaClO. Afterwards, embryos were carefully sliced, and plumules were taken out and were inoculated on callus induction media following method established by (Neema *et al.,* 2022; Bhavyashree *et al.*, 2016).

Three amino acids (glutamine 200 mg/L, L-asparagine-100 mg/L, and proline-100 mg/L) and one organic additive compound, *i.e.* casein hydrolysate

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(250 mg / L) were added individually or in combinations with basal medium (M) to form six different combinations along with 2, 4-D (16.5 mg / L), sucrose (3%), charcoal (0.1%) and agar (0.8%), *i.e.* MC1 to MC6 [MC1: M+2, 4-D-16.5 mg / L + 30 g / L sucrose+ activated charcoal- 1000 mg / L + agar 0.8%; MC2: M+ 2,4-D-16.5 mg / L+ glutamine-200 mg / L+ 30 g / L sucrose + activated charcoal- 1000 mg / L + agar 0.8%; MC3: M + 2, 4-D-16.5 mg / L + glutamine-200 mg / L+ casein hydrolysate -250 mg / L+ 30 g / L sucrose + activated charcoal- 1000 mg / L+ agar 0.8%; MC4: M + 2,4-D-16.5 mg / L+ casein hydrolysate -250 mg / L + 30 g / L sucrose+ activated charcoal 1000 mg / L+ agar 0.8%; MC5: M +2, 4-D-16.5 mg / L+ L-asparagine-100 mg / L+ proline 100 mg / L+ 30 g / L sucrose+ activated charcoal- 1000 mg / L+ agar 0.8%; MC6: M + 2,4-D 16.5 mg / L + L-Asparagine-100 mg / L + proline 100 mg / L + glutamine 200 mg / L + casein hydrolysate 250 mg / L+ 30 g / L sucrose+ activated charcoal 1000 mg / L+ agar 0.8%; MC: media combinations 1 to 6 and M: basal medium].

Each of the above combinations were tried with three basal medium, *i.e.* Murashige and Skoog (MS), 1 / 2MS (half-strength MS medium) and Eeuwens (1976) Y3 medium to form 18 different treatment combinations. ICAR-CPCRI Standardized media for callus initiation from coconut plumule *i.e.,* Tc: Eeuwen (1976) Y3 basal media +16.5 mg / L 2, 4 -D + 1 mg / L TDZ + 1000 mg / L charcoal + 0.8% agar was considered as a standard for comparing all 18 treatments (Rajesh *et al.,* 2014). All the treatment combinations of media were prepared and autoclaved at 121°C and 1.06 kg / cm^2 pressure for 20 minutes after adjusting pH to 5.8. Each treatment with along with control was replicated thrice and a total of 57 plates were inoculated, each with 10 WCT plumules and was kept under dark condition.

After a month, plumules from each treatment plate were subcultured into individual test tubes (50 ml test tubes containing 15 ml solid media) containing same media composition of respected treatments with reduced 2,4-D concentration (10 mg / L). These inoculated cultures were maintained in continuous dark condition for calloid tissue initiation and multiplication. Observations on plumule bulging, browning / necrosis, the formation of calloid clump and embryogenic calloid production were recorded on 7^{th} day, 10^{th} day, 15^{th} and 45^{th} days after inoculation, respectively.

Percentage of explant browning (number of browned plumules out of the total number of plumules inoculated), explant bulging (% plumular bulging) and formation of callus clumps were calculated and recorded for each treatment. Embryogenic calloid production and quantity of calloid tissue from each in response to different media combinations were tested based on visual measurements since the size of the explant is too small ($\leq$ 1 mm) and its callusing capacity is found to be minimal. Measurements on explant and its response to callusing were taken on five different visual scales *i.e.* scale 0: plumules without bulging (plumule with browning) or no callusing, scale 1: bulged plumule without callusing, scale 2: bulged plumule with slow multiplication symptoms with initiated callus nodules, scale 3: bulged plumules with extended callus nodules, scale 4: bulged plumules with good callus growth with callusing nodules, scale 5: bulged plumules with high calli growth with extended callus nodules.

The values were individually observed for each replication, and the total value was averaged by dividing with the number of functional plumules present in that replication (functional plumules: plumules without browned or contaminated). The results were analyzed using SAS software and means were compared by Duncan's Multiple Range Tests (DMRT).

RESULTS AND DISCUSSION

Symptoms of browning or necrosis of explant tissues was noticed after a week of inoculation in a callus initiation medium. In different treatments, explant browning was ranged 3.3% to 30% (Table 1). Media combinations were taken into consideration; MC2 displayed minimal browning of 9.99%, followed by MC1 and MC3 with 14.44% and 17.77% respectively. While highest browning of 25.55% was noticed in MC4 combination with all three basal media. The additional browning observed in MC4 combination might have triggered by the organic supplement 'casein hydrolysate'.

However, when casein hydrolysate was combined with other amino acids such as L-glutamine in MC3, or L-asparagine, proline, glutamine in MC6 combinations, percentage browning was reduced to 9.99% and 18.88% respectively from 25.55%. Though considerable browning was noticed with the addition of casein hydrolysate to the medium, it does not kill the explant tissue fully; instead, calloid tissue formation was seen on temporarily browned explant tissue. Casein hydrolysate is a complex, undefined natural organic substance popularly used in cell culture medium (Bhatia, 2015).

Organic substances are active sources of several amino acids, hormones, vitamins, fatty acids, carbohydrates, and several plant growth substances (Bhatia, 2015). Hydrolysates carry a rich amount of glutamine, proline, and lysine, along with other plant growth substances (Wang *et al.,* 2013). The positive influence of casein hydrolysate especially for callus initiation and multiplication was reported in several

Table 1. Browning (%) and embryogenic calloid production from plumular explants of coconut using different amino acids with three different basal media

Media	Treatment	Browning (%)	Plumular bulging and formation of callus clump (%)	Embryogenic callus (scale 0-5)
Control	T_c	13.33	86.66	1.60 DCEB
MS	T_1	13.33	80.00	1.41 DCEF
	T_2	16.66	83.33	1.58 DCEB
	T_3	23.33	76.66	1.09 EF
	T_4	26.66	73.33	1.29 DCEF
	T_5	26.66	73.33	1.17 DEF
	T_6	30.00	83.33	1.73 DCB
Y3	T_7	13.33	86.66	1.60 DCEB
	T_8	10.00	90.00	2.43 A
	T_9	10.00	90.00	2.00 AB
	T_{10}	23.33	76.66	1.00 F
	T_{11}	13.33	86.66	1.84 CB
	T_{12}	16.66	70.00	1.19 DEF
1/2 MS	T_{13}	16.66	83.33	1.48 DCEBF
	T_{14}	3.33	96.66	1.75 DCB
	T_{15}	20.00	80.00	1.00 F
	T_{16}	26.66	73.33	1.36 DCEF
	T_{17}	16.66	80.00	1.18 DEF
	T_{18}	13.33	86.66	1.38 DCEF
	SE	9.55	9.79	0.24
	CD (5%)	NS	NS	0.48

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plant species (Cai *et al.*, 2013; Okumoto *et al.*, 2016; Murkute, 2020).

Of three tested basal media, used highest explant browning or necrosis was observed with MS media (21.99%) which was followed by 1/2MS (16.1%) and least browning was observed in combinations with Y3 basal media (14.44%). The superiority of Y3 basal media over MS was also proven by Muniran *et al.* (2008) for direct and indirect regeneration in oil palm. When basal media and additional media combinations were compared to highest browning of 30% was noticed in treatment T4, which had a combination of MS basal media with MC4 additional component 'casein hydrolysate'.

Increase in size of plumules (bulging) was noticed after 5, 7 days after inoculation. These plumular tissues further gave rise to calloid clumps instead of germinating into individual plantlet due to the higher concentration of the 2, 4-D, *i.e.* 16.5 mg/L. Plumular bulging, and clump formation were noticed in all 19 treatments, including control. In various treatments, this parameter varied from 70 to 96%. The highest percentage of bulging and calloid formation was observed in treatment containing 1/2MS media with MC2 combination where glutamine was present alone instead of different amino acids combination. This was followed by T_8 and T_9 with 90%, where Y3 media was present along with MC2 (Y3 + 2,4-D 16.5 mg/L+ glutamine 200 mg/L) and MC3 (MC3-Y3 + 2, 4-D 16.5 mg/L + glutamine200 mg/L + casein hydrolysate 250 mg/L) combination.

Considerable increase in calloid formation was noticed with the addition of glutamine to the Y3 and 1/2MS media, while the slightly lower response was noticed in MS basal media. Supple-mentation of basal media with glutamine facilitates higher nitrogen uptake by the tissue due to an increase in assimilation capacity as well as a nitrogen source (Okumoto *et al.*, 2016). Callus induction from immature cotyledons and embryos of walnut was enhanced with the use of glutamine (Cai *et al.*, 2013). Influence of glutamine on dedifferentiation and dedifferentiation process of cell cultures process was also noticed (Habib *et al.*, 2015).

Embryogenic calloid formation was observed from the plumular explants after 30 days of inoculation into the callus induction media. Calloid tissue from the explants was subsequently transferred to a media containing a reduced amount of 2, 4-D (10 mg/L) with the same media combinations. Highest percentage of embryogenic calloid production was obtained from the

treatment T_8 (2.42) containing Y3 media with MC2 combination (MS +2, 4-D-16.5 mg/L+ glutamine 200 mg/L) which was followed by treatment T_9 with the visible value 2 consists of Y3 basal media with additional glutamine (Y3+2, 4-D 16.5 mg/L+ glutamine 200 mg/L+ casein hydrolysate 250 mg/L) followed by T_{11} (Y3 +2, 4-D 16.5mg/L+ L-asparagine 100 mg/L+ proline 100 mg/L) and T_{14} (1/2MS +2, 4-D 16.5mg/L+ glutamine 200 mg/L) with the values 1.84 and 1.75 respectively.

The increase in calloid production in treatments T_8, T_9, T_{11} and T_{14} over control was 34%, 20%, 13% and 9% respectively and all four treatments were found to have a common additional nitrogen additive 'glutamine' with Y3 basal media. Previous studies have been reported that the addition of glutamine to the medium containing auxins and cytokinins or cytokinin's alone boosted the regenerative ability of the callus in wheat by 6 to 10%. Inclusion of glutamine (34.2 µM) enhanced the cell count in coconut suspension (Bhavyashree *et al.*, 2016).

Absence of glutamine in the callus tissue leads to the loss of embryogenic capacity in Cryptomeria japonica callus cultures (Ogita *et al.*, 2001). However, the positive influence of amino acids, asparagine and proline has been reported in several studies (Suekawa *et al.*, 2019), the influence of these amino acids (*i.e.*, asparagine and proline) in aforesaid concentrations had shown less response in coconut calloid production and multiplication in the present study.

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CONCLUSION

Addition of amino acid glutamine (200 mg/L) to 2, 4-D (16.5%) positively influenced the calloid production from plumular explants of coconut. Trails on other amino acids with lower and higher concentrations may provide detail insight into the effect of embryogenic calloid production from plumular explants of coconut. Though all the additional components act as a nitrogen source to the media, glutamine exhibited higher response as compared to other tested compounds.

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