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Effects of cultivar, culture medium, and plasma treatment on callus induction in *Capsicum* spp. through *in vitro*

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Abstract Four chili pepper cultivars were successfully germinated on MS medium under *in vitro* conditions. PEP12 cultivar showed the highest germination speed and rate throughout all weeks, while ANT4 cultivar had a low germination rate in the 2nd week but an increased rate in the 3rd week. PEP6 cultivar had the lowest germination rate in the 3rd week, and YS cultivar had a moderate germination rate in all weeks. Callus derived from the hypocotyl of ANT4 on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin showed the highest callus induction efficiency (67.38 mm^2). The callus induction experiment using air plasma jet treatments on PEP12 and YS revealed that PEP12 exposed to plasma for 9 seconds exhibited the highest callus growth rate at both the 2nd and 4th weeks. In contrast, YS consistently showed lower growth than PEP12 under all conditions. However, plasma treatments, even at the maximum exposure of 12, was not significantly differed as compared to the control group, indicating that plasma had only a minimal impact on callus growth, with most observed variations attributable to cultivar effects.

Keywords: Callus, Chili pepper, Plasma treatment, Tissue culture, Callus induction

Introduction

Chili pepper (*Capsicum* spp.), belonging to the Solanaceae family, is an economic crop widely consumed in daily life. However, limited chili pepper production leads to fluctuating high market prices. Chili pepper production is adversely affected by diseases and insect pests. Control of diseases or pests using cultivation techniques and chemical treatments hasn't had satisfactory results yet. Therefore, breeding chili pepper is important for developing new cultivars with

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improved agronomic traits, such as high yield, resistance to diseases and insects, and fruit quality. Nevertheless, conventional breeding methods have limitations due to being time-consuming and limited genetic diversity for developing new varieties with desired characteristics (Akhatar *et al.*, 2022). Consequently, biotechnology, such as tissue culture has been introduced to assist in breeding efforts.

The tissue culture reduces contamination by pathogens and fungi, which affects seed germination and seedling development. *In vitro* seed germination of chili pepper seeds is also used for propagating rare or economically important chili cultivars, causing production of high-quality, disease-free seedlings (Smith and Drew, 1990). Moreover, seedlings derived from *in vitro* seed culture can be subsequently used for breeding purposes, specially callus induction, such as mutation induction, which is important information for chili pepper breeding and commercial production. Callus induction is important step in plant tissue culture as it provides the material for regenerating new plants and a resource for genetic improvement. Liu *et al.* (2018) reported that callus induction is an effective tool for de novo plant regeneration. Several factors influence callus induction, including the components and concentrations of plant growth regulators in the culture medium. The success of plant tissue culture hinges on the selection of the type and concentration of plant growth regulators in the culture medium (Shi *et al.*, 2014). These growth regulators are important for growth and development (Huang *et al.*, 2012). In tissue culture, auxins and cytokinins can stimulate callus induction and the differentiation of cells into new organs (Barreto *et al.*, 2010; Feng *et al.*, 2010). The principle that a low cytokinin with higher auxin induces root formation, and a high cytokinin with low auxin induces shoot formation is the fundamental basis of interaction in plant tissue culture. Due to its widespread success and application, many types of plant medium protocols have been reported (Su *et al.*, 2011), such as the placental culture of chili pepper on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin was found to be the most effective for callus induction (Kabita *et al.*, 2019). Callus induction from hypocotyl of chili pepper in MS medium supplemented with 2.5 mg/L NAA and BAP (Kumar *et al.*, 2017) or 2.5 mg/L NAA + 2.5 mg/L IAA and 3.5 mg/L 2.4 D + 0.25 mg/L TDZ (Yunita *et al.*, 2021) and callus induction from leaf of *Capsicum annum* with MS medium supplemented with B5 vitamins and 1 mg/L 2,4D (Kim and Lim, 2019). Auxins and cytokinins are commonly used hormones for callus induction and regeneration. However, callus growth can be limited by various factors such as plant species, environmental conditions, and growth regulators themselves (George *et al.*, 2008). Therefore, new methods are being sought to induce callus growth to enhance the ability of plant tissue culture.

Plasma technology is gaining increasing interest at present. Plasma is the fourth state of matter, comprising ions, electrons, and neutral particles. Plasma can be generated by supplying energy to a gas until it ionizes (Fridman, 2008). Plasma possesses unique chemical and physical properties that allow for its application in various fields, such as sterilization and plant growth stimulation (Laroussi and Lu, 2005). Chemical and physical seed priming is an effective approach to meet agricultural demands by promoting early growth and enhancing resistance to environmental stress (Hu *et al.*, 2016; He *et al.*, 2017; Sheteiwy *et al.*, 2022). Diverse seed priming techniques offer numerous benefits, including the control of microbial populations on the seed surface, accelerated germination (Lee *et al.*, 2021), enhanced growth (Zhao *et al.*, 2021), improved water and nutrient absorption efficiency (Rasooli *et al.*, 2021), stimulated metabolic processes, and increased stress tolerance (Li *et al.*, 2021), all of which contribute to reduced production cost. There is a report support the notion that plasma seed priming is an effective, environmentally friendly, and cost-efficient method. For example, plasma seed priming has demonstrated advantages in various plant species, including chrysanthemum (Skoro *et al.*, 2022), *Bidens pilosa*, *Solanum nigrum*, and *Trifolium repens* (Zhao *et al.*, 2021), ginseng (Lee *et al.*, 2021), buckwheat (Mravlje *et al.*, 2021), and *Cuminum cyminum* (Rasooli *et al.*, 2021). However, the most research was focused on seed priming, with relatively limited exploration of plasma technology's application in plant cell and tissue culture. Therefore, the objective of this study was to evaluate the effects of cultivar, culture medium, and plasma treatment on callus induction in *Capsicum* spp. through in vitro.

Materials and methods

Seed culture

Four *Capsicum annuum* chili pepper seed varieties were used: 1) Yodson (commercial cultivar), 2) ANT4 (anthracnose resistant cultivar), 3) PEP6 (pepper yellow leaf curl virus resistant cultivar), and 4) PEP12 (pepper yellow leaf curl virus resistant cultivar). These seeds were then washed by allowing water to flow through them for 30 minutes. They were then sterilized by sequential soaking in 70% ethanol for 1 minute and 15% (v/v) sodium hypochlorite for 20 minutes, followed by 3 washes in sterile distilled water for 5 minutes each. The seeds were placed in jars on MS medium (Murashige and Skoog, 1962) and incubated in the dark at 26 ± 2 °C for 3 weeks. The total number of seeds in each culture bottle and the number of germinated seeds were recorded every 7 days to compare the germination speed of each chili pepper variety. Seed germination percentage was

calculated using the formula: germination rate (%) = (Number of germinated seeds / Total number of seeds) x 100. Seedlings were used in the next experiment.

Callus induction

Seedlings obtained from an aseptic seed culture were excised into explants, namely cotyledons and hypocotyls. Cotyledons were prepared by removing the base and tip of the leaf, while hypocotyls were cut into 1 cm long segments. Both types of explants were cultured on two formulations of MS medium: 1) MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BA, and 2) MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin. The experiment was designed as a 4x2x2 factorial in a completely randomized design. Callus induction was quantified by measuring callus size in mm^2. Callus color and morphology were also recorded weekly for all explants.

Plasma induced

Callus induced from PEP12 and Yodson (YS) were cut into 5.0 x 5.0 mm. pieces and cultured on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin. A 2x5 factorial experiment in a completely randomized design was implemented; the mobile plasma jet (MPJ®) was placed 8 cm away from the explants. Plasma was applied for durations of 0, 3, 6, 9, and 12 seconds. Its properties were examined through optical emission spectroscopy in the 200–900 nm range to identify reactive oxygen and nitrogen species. Callus induction percentage was calculated relative to the initial explant size, and callus color was recorded weekly for all explants. Callus size increase (%) = [(final callus size – initial callus size) / initial callus size] x 100.

Results

Seed culture

Four chili pepper cultivars were cultured under *in vitro* conditions. The germination rates of the seeds showed significant differences. In the first week, PEP12 exhibited the highest significant germination rate, with an average of 12.14%, indicating the fastest germination speed. In contrast, ANT4 had the lowest germination rate at 0.00%, while PEP6 and YS showed moderate rates of 0.95% and 3.84%, respectively. By the second week, the germination rates of all chili pepper cultivars significantly increased. PEP12 maintained the highest germination rate at 40.51%, followed by YS at 26.40%, and PEP6 at 21.11%.

ANT4 still had the lowest rate at 17.34%. Upon reaching the third week, the germination rates continued to increase across all cultivars. PEP12 remained the highest at 88.33%, closely followed by ANT4 at 85.14%, and YS at 76.48%. PEP6 recorded the lowest germination rate this week at 69.82% (Figure 1). However, in the third week, the germination rates of ANT4 and PEP12 were not significantly different (Table 1). Experimental results indicate that cultivar significantly affected the germination rate and germination speed of chili pepper seeds.

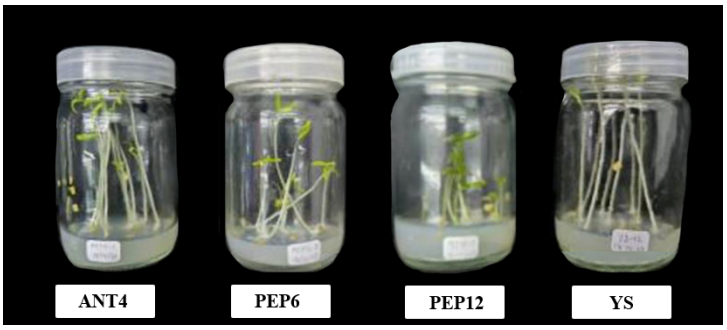

Figure 1. Seedlings derived from seed culture on MS medium after 3 weeks

Table 1. Germination rates of four chili pepper cultivars cultured under *in vitro* conditions

Genotype	Germination (%)		
	Week 1	Week 2	Week 3
ANT4	0.00^{c}	17.34^{c}	85.14^{ab}
PEP6	0.95^{bc}	21.11^{c}	69.82^{c}
PEP12	12.14^{a}	40.51^{a}	88.33^{a}
YS	3.84^{b}	26.40^{b}	76.48^{bc}
F-test	**	**	**
C.V. (%)	54.67	13.16	8.35

** Significant different at $P \leq 0.01$; Means within column followed by the same later are not significant different as determined by LSD (least significant difference)

Callus induction

The cotyledon and hypocotyl fragments of four chili pepper cultivars are ANT4, PEP6, PEP12, and YS cultured on MS medium supplemented with 2 mg/L 2,4-D with 0.5 mg/L BA and 2 mg/L 2,4-D with 0.5 mg/L kinetin, it was found that ANT4 had the highest callus size in every week of culture, with a callus size of 51.09 mm^2 in the 3rd week, while the callus size of the PEP6, PEP12 and YS were no significant different (Table 2). The most medium for inducing

callus (41.37 mm^2) was the MS medium supplemented with 2,4-D 2 mg/L and kinetin with 0.5 mg/L.

Table 2. Effects of different chili pepper cultivars, culture medium, and explants on callus size

Genotype	Callus size (mm^2)		
	Week 1	Week 2	Week 3
ANT4	4.24[a]	22.59[a]	51.09[a]
PEP6	3.41[b]	16.34[b]	27.95[b]
PEP12	2.99[b]	16.92[b]	30.14[b]
YS	2.38[c]	11.45[c]	26.71[b]
F-test	**	**	**
Plant growth regulators			
2,4-D + BA	3.83[a]	15.45[b]	26.57[b]
2,4-D + Kinetin	2.68[b]	18.20[a]	41.37[a]
F-test	**	**	**
Explant			
Cotyledon	5.39[a]	21.39[a]	37.63[a]
Hypocotyl	1.12[b]	12.26[b]	30.31[b]
F-test	**	**	**

** Significant different at $P \leq 0.01$; Means within column followed by the same later are not significant different as determined by LSD

The combined effects of chili pepper cultivar, culture medium, and explants that resulted in the highest callus size weeks at 1 and 2 were the cotyledon culture of ANT4 on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BA (10.04 and 34.40 mm^2), which showed the fastest callus. However, at week 3, the hypocotyl of ANT4 cultured on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin was the most effective in inducing callus (67.38 mm^2). The result showed that 100% callus formation in every treatment. While week 1 of culture showed the lowest callus (Table 3), week 3 of culture showed the fastest callus. Moreover, callus derived from cotyledon and hypocotyl cultures was white and yellow, respectively, and all the calluses were compact callus (Figure 2).

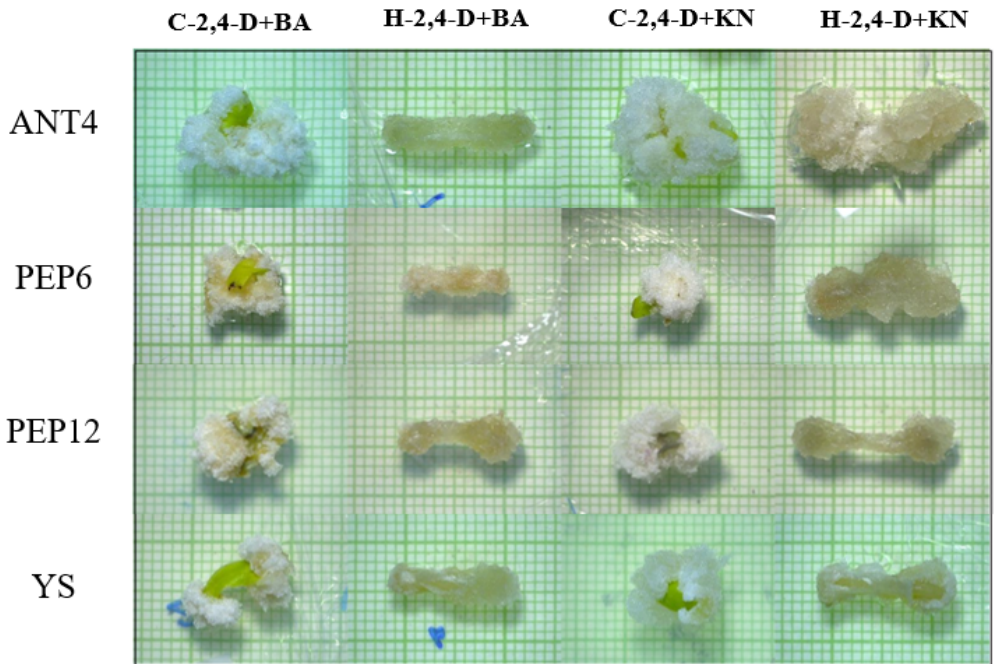

Figure 2. Callus development from different cultivars (ANT4, PEP6, PEP12 and YS), explant (C = cotyledon, H = hypocotyl) and MS medium supplemented with 2,4-D, + BA or 2,4-D + KN after 3 weeks

Effect of plasma on callus induction

The optical emission spectrum of the air plasma jet exhibits several characteristic peaks associated with reactive oxygen and nitrogen species (RONS (Figure 3)). The NO A–X (γ) band was observed in the ultraviolet range (200–300 nm), and a clear OH emission at 308 nm. Additionally, strong emission lines of N_2 occurred at 336.61 nm, 357.17 nm (2nd positive band), and 379.99 nm (1st negative band). A notable atomic oxygen line was detected at 777.4 nm. These gas-phase RONS dissolved into media, forming NO_2^- and NO_3^-, enriching plasma-activated media (PAM) with bioaccessible nitrate that supports plant growth.

Table 3. Effects of chili pepper cultivar, culture medium, and explant type on callus size

Genotype (A)	PGR (B)	Explant (C)	Callus formation (%)	Callus size (mm^2)	Color	Texture
ANT4	2,4-D + BA	C	100	53.66^{ab}	white	Compact
		H	100	30.15^{d-f}	Yellow	Compact
	2,4-D + KN	C	100	53.15^{b}	white	Compact
		H	100	67.38^{a}	Yellow	Compact
PEP6	2,4-D + BA	C	100	27.13^{e-g}	white	Compact
		H	100	17.59^{fg}	Yellow	Compact
	2,4-D + KN	C	100	34.35^{c-e}	white	Compact
		H	100	41.48^{b-d}	Yellow	Compact
PEP12	2,4-D + BA	C	100	25.57^{e-g}	white	Compact
		H	100	15.33^{g}	Yellow	Compact
	2,4-D + KN	C	100	45.26^{bc}	white	Compact
		H	100	25.64^{e-g}	Yellow	Compact
YS	2,4-D + BA	C	100	27.82^{e-g}	white	Compact
		H	100	15.30^{g}	Yellow	Compact
	2,4-D + KN	C	100	34.07^{c-e}	white	Compact
		H	100	29.63^{d-f}	Yellow	Compact
F-test A				73.03^{**}		
F-test B				121.02^{**}		
F-test C				29.55^{**}		
F-test A x B				1.55^{ns}		
F-test A x C				4.78^{**}		
F-test B x C				24.35^{**}		
F-test A x B x C				13.23^{**}		
C.V. (%)				13.72		

BA= 6-benzylaminutesopurine, KN = kinetin, C = cotyledon, H = hypocotyl

** Significant different at $P \leq 0.01$ and ns Non-Significant; Means within column followed by the same later are not significant different as determined by Tukey's HSD (Honestly Significant Difference)

The combined effects of chili pepper cultivars and air plasma treatment have a significant impact on callus growth rate. In both the 2^{nd} and 4^{th} weeks, PEP12 was treated with plasma for 9 seconds, resulting in the highest growth rates (119.23% and 315.60%, respectively). Conversely, YS consistently exhibited notably lower growth across all conditions (Table 4). The duration of plasma exposure significantly influenced callus growth in PEP12 but was not affected YS. Additionally, a notable interaction between genotype and plasma exposure time was detected at week 4.

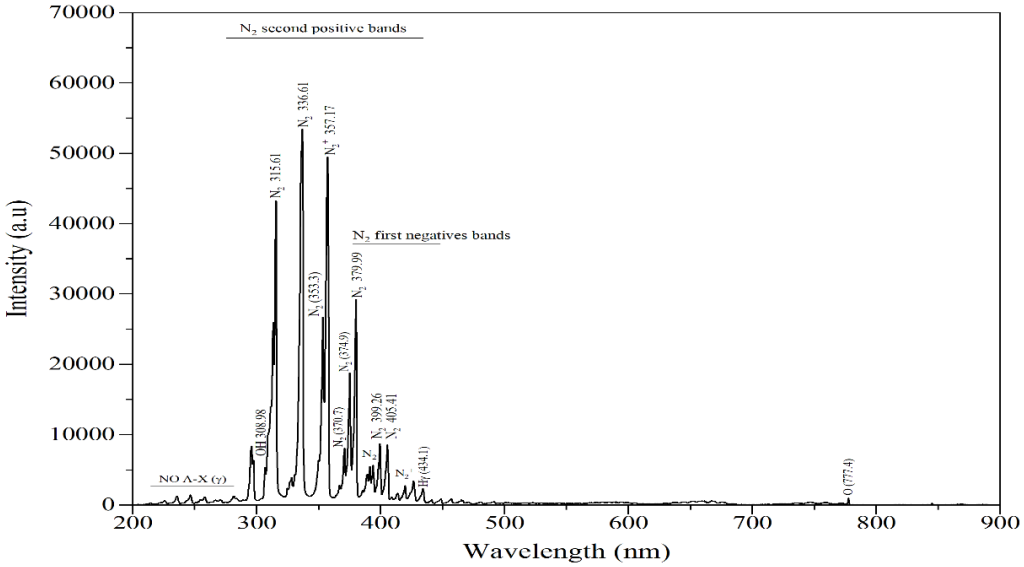

Figure 3. Optical emission spectrum of the air plasma jet showing NO, OH, N_2, and O peaks

Table 4. Effects of genotype, and plasma exposure time on callus formation after culture on MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin in 4 weeks

Genotype (A)	Exposure time (s) (B)	Callus formation (%)[1]		Color
		Week 2	Week 4	
PEP12	0	68.85^{bc}	263.66^{a}	Mauve brown
	3	46.04^{c}	155.98^{c}	Mauve brown
	6	71.85^{bc}	246.13^{ab}	Mauve brown
	9	119.23^{a}	315.60^{a}	Mauve brown
	12	90.10^{ab}	261.09^{a}	Mauve brown
YS	0	61.27^{bc}	174.14^{bc}	Yellow
	3	58.95^{bc}	178.05^{bc}	Yellow
	6	51.93^{bc}	171.49^{bc}	Yellow
	9	71.41^{bc}	164.98^{c}	Yellow
	12	62.45^{bc}	181.09^{bc}	Yellow
F-test A		4.91*	23.42**	
F-test B		3.26*	2.50^{ns}	
F-test A x B		1.55^{ns}	3.24*	
C.V. (%)		31.71	19.97	

[1] Compared to the size of callus before plasma treatment (day 0)

** Significant different at $P \leq 0.01$, * Significant different at $P \leq 0.05$ and ns Non-Significant; Means within column followed by the same later are not significant different as determined by Tukey's HSD

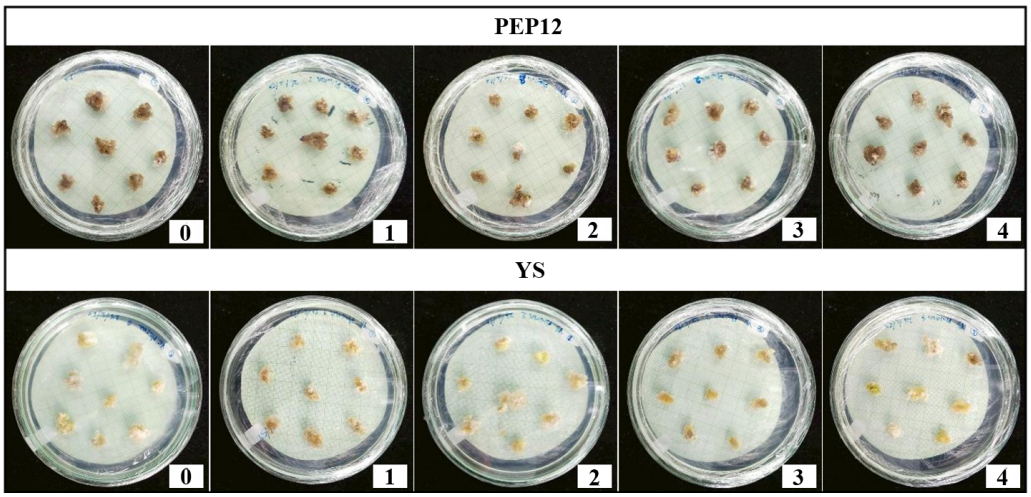

Figure 4. Callus formation in PEP12 and YS cultivars after 4 weeks, with plasma treatments for 0, 3, 6, 9, and 12 seconds

Callus growth after treatment with an air plasma jet did not significantly differ from that of the control group (without plasma treatment). The observed variation in callus growth was attributable to differences in chili pepper cultivars. The callus of PEP12 appeared mauve brown, whereas YS callus was yellow, indicating distinct chemical compositions between the two cultivars. In PEP12, high levels of anthocyanin synthesis matched the purple coloration of stems, leaves, flowers, and young fruits. Consequently, some callus produced anthocyanin, resulting in partial purple coloration (Figure 4).

Discussion

In vitro cultivation of chili seeds shows that chili pepper cultivars have different effects on germination rate and speed (Miah *et al.*, 2008; Akinyosoye *et al.*, 2014) under the same culture conditions. Callus from cotyledon and hypocotyl of four chili pepper cultivars (ANT4, PEP6, PEP12, and YS) cultured on the MS medium showed that ANT4 had the highest callus size in every week of culture, while PEP6, PEP12, and YS exhibited no significant difference in callus size. This aligns with previous findings that chili genotypes affect callus growth (Shafiq *et al.*, 2022). The most effective medium for callus induction was MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin (Kabita *et al.*, 2019), with hypocotyl explants proving to be the most effective for callus induction. In addition, callus from cotyledon and hypocotyl culture resulted in white and yellow callus, respectively. While Kasim *et al.* (2023) reported that cotyledon responded better than hypocotyls, producing white, greenish-white,

yellow or yellowish-white callus and hypocotyl producing white callus. The type of callus growth *in vitro* is determined by the ratio of cytokinin to auxin. The formed callus was compact, unlike the friable callus induced by Umamaheswari and Lalitha, (2007) in their study on *C. annuum* chili groups using MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L kinetin. Using air plasma treatments to enhance callus induction efficiency revealed significant differences from the control after 2 weeks. However, by week 4, these differences were no longer significant. The indicated that the plasma had a minor impact on callus growth under these conditions, with the primary influence coming from the chili pepper cultivar.

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Conflicts of interest

The authors declare no conflict of interest.

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