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Optimizing Callus Induction in Guar (*Cyamopsis tetragonoloba* L.): Impact of Hormonal Compositions and Explant Types

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ABSTRACT

Callus induction and regeneration can greatly help in improving the progress of genetic modification and help in gene transfer. Accordingly, this study investigated the optimal explant and growth regulators for callus formation. The experiment was performed in a factorial experiment based on a completely randomized design in 5 replications with hormonal composition (13 levels) and explant types (rootlet, leaf and stem) as factors. The levels of hormonal composition contained NAA, BAP, 2,4-D and TDZ applied to the MS media. After 80 days, the explants were measured for various traits, including days to callus formation, callus formation percentage, fresh and dry weight, and mass moisture percentage. According to the analysis of variance, hormonal composition, the type of explant, and their interaction had significant effects on fresh and dry weight and callus formation. [2,4-D (2 mg L^{-1}) + TDZ (2 mg L^{-1})] and [NAA (2 mg L^{-1}) + BAP (1 mg L^{-1})] treatments had the highest and lowest percentage of callus formation, respectively. The correlation test revealed a significant relationship between callus formation and both fresh weight and mass moisture percentage. However, callus formation did not show a significant correlation with dry weight. In general, treating leaves with the TDZ resulted in the highest dry and fresh weights compared to treating roots with the BAP. To enhance callus production and achieve better weight in this plant, it is recommended to use leaf explants combined with TDZ (2 mg L^{-1}) and 2,4-D (2 mg L^{-1}).

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1. Introduction

Guar (*Cyamopsis tetragonoloba* L.) is a one-year summer plant that is resistant to drought and soil salinity. By fixing atmospheric nitrogen and adding its residues to the soil, this plant increases nitrogen and soil organic matter (Singla *et al.*, 2016). Guar is a self-pollinating plant from the *Fabaceae* family (Malani *et al.*, 2024), which is also known as cluster bean. It can be used to establish a low-input agriculture system. In the case of explaining the ecological advantages and numerous benefits of guar for agricultural systems, as well as the major industrial and medicinal uses of its seed products, along with the possibility of using plant shoots as fodder and green manure (Gresta *et al.*, 2014). Guar gum, sourced from the seeds' endosperm, has recently become a significant economic product, consisting of approximately 90% galactomannans. Guar gum is a natural edible thickening agent (Perchyonok *et al.*, 2016), and as such, it finds extensive utilization across a wide range of industries including textiles, paper, oil, explosives, cosmetics, and pharmaceuticals (Awan *et al.*, 2024). This affordability and versatility make it a popular choice among manufacturers and producers looking for effective thickening agents. Additionally, Guar gum is used as a binding agent in tablet and liquid formulations, as well as a strengthening agent, stabilizer, emulsifier and suspending agent. The medicinal properties of guar gum include reducing blood sugar, antimicrobial properties, reducing appetite and reducing fats, especially cholesterol. Also, it is used in the cosmetics industry (Thombare *et al.*, 2016). Guar gum is considered an important substrate in the oil field. It's used to boost production and efficiency in

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the oil and gas industry. Nowadays, its biggest application is in the oil and gas sector. Guar gum increases oil recovery rates in oil fields and helps improve oil production. As a controlling agent, it can activate oil derivatives in an active oil field. Guar gum is a leading contributor to oil processes. Guar gum and its derivatives are used effectively to thicken water, even at low concentrations, in the fracking of oil and gas wells. The universal demand for guar has increased recently, leading to its introduction in countries like South Africa, Australia, and Brazil, each with diverse climates and seasons (Benakanahalli *et al.*, 2021). This creates a need to develop enhanced guar varieties through breeding programs suited for different climatic conditions. Additionally, it is crucial to establish propagation methods beyond seeds, such as tissue culture, to support these breeding efforts.

A study investigated the use of guar gum as a gelling agent in both microbial and plant tissue culture media. The study compared the effects of guar gum on the growth and differentiation of cultured plants with those of other well-known gelling agents. Results showed that guar gum outperformed the other agents in promoting various morphogenic responses. Besides being cost-effective, these gelling agents are biocompatible and biodegradable (Rani *et al.*, 2025).

Cultivation and exploitation of this plant will increase in the agricultural ecosystems of the country. The high commercial value of guar seed is mainly due to the extraction of endospermic gum (galactomannan) from it (Verma *et al.*, 2024), which is commercially known as digestive gum (Matsumiya *et al.*, 2024). Various industrial applications of guar gum can be listed so that any product in which water is one of the components (food, pharmaceutical, textile, cosmetics and in the industries of drilling oil and gas wells and extracting from guar gum can be used in mines (Malani *et al.*, 2024). Following this increase in demand, the price of guar has increased by about 230% and even more (Gresta *et al.*, 2014).

Ahlawat (2022) studied the tissue culture of genotypes of this plant and observed several direct stem regenerations from hypocotyls in culture medium with 1 mg L^{-1} of benzylaminopurine (BAP) in *C. serrata*. The cotyledon nodule produced multiple shoots on MS medium with 2 mg L^{-1} BAP. *C. senegalensis* displayed direct branching only from hypocotyl explants in 1 mg L^{-1} BAP medium. Maximum callus induction from cotyledon explants occurred in *C. serrata* and *C. senegalensis*, while ½ MS supplemented with 0.5 mg L^{-1} and 1 mg L^{-1} IBA promoted the best rooting in *C. senegalensis* and *C. serrata*. Ahmad et al. (2013) noted that the optimum response for shoot regeneration was on 1.5 mg L^{-1} BAP, and the highest shoot number was achieved with 1 mg L^{-1} Thidiazuron (TDZ).

Plant propagation, whether through reproductive or vegetative techniques, encounters various challenges. In reproductive methods, seeds might be damaged or result in genetically diverse offspring. Meanwhile, vegetative methods like planting, grafting, and cutting tend to be slow and costly. Cultivation in controlled medium, such as greenhouses or laboratories, offers a more efficient alternative. Tissue culture techniques excel over traditional approaches by enabling exact control of physical and chemical medium conditions. This method allows for the production of numerous genetically identical specimens. Understanding tissue culture across a variety of plants, including non-agricultural species, can aid breeders in improving plant characteristics, transferring beneficial genes, and preserving new varieties. Research on tissue culture for guar is limited, with no studies conducted in Iran.

2. Materials and methods

This study aimed to investigate the formation of callus in guar plants under the influence of different hormonal compositions and explant types. This study utilized a Pakistani ecotype of guar and was conducted following a factorial arrangement in a completely randomized design. The experiment included five replications, thirteen different hormone compositions, and three types of explant treatments. Prior to the main experiment, a pre-test was conducted based on information from previous trials to determine the most effective hormone treatments. At first, in order to remove external contamination, seeds were washed once for 30 s in 70% ethanol alcohol and then twice were rinsed with distilled water, and the seeds were treated for ten minutes with sodium hypochlorite solution of 10 % and then washed with distilled water in three times. Then we placed ten seeds for germination in jars containing 1/2 MS (Murashige, and Skoog, 1962) (Fig. 1) and after two weeks of germination, the desired explants (rootlet, leaf and stem) were separated under a laminar hood and put in petri-dishes that containing hormonal treatments with

different compounds of Naphthyl acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), BAP and TDZ (Table 1).

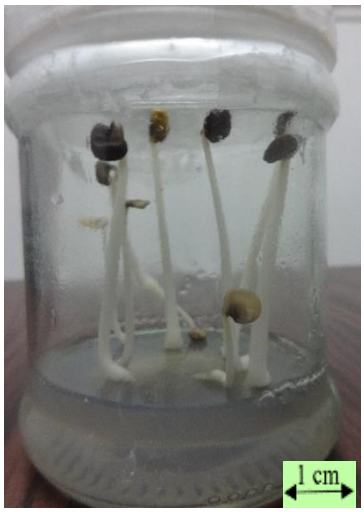

Figure 1. Cultivation of sterilized guar seeds on MS culture medium to produce suitable *in vitro* explants.

Table 1. Hormonal compounds used in the culture medium

	Treatment (Hormones)
1	MS + Without hormones (Control)
2	MS + NAA (1 mg L^{-1}) + BAP (1 mg L^{-1})
3	MS + NAA (2 mg L^{-1}) + BAP (1 mg L^{-1})
4	MS + NAA (2 mg L^{-1}) + BAP (2 mg L^{-1})
5	MS + 2,4-D (1 mg L^{-1}) + BAP (1 mg L^{-1})
6	MS + 2,4-D (2 mg L^{-1}) + BAP (1 mg L^{-1})
7	MS + 2,4-D (2 mg L^{-1}) + BAP (2 mg L^{-1})
8	MS + NAA (1 mg L^{-1}) + TDZ (1 mg L^{-1})
9	MS + NAA (2 mg L^{-1}) + TDZ (1 mg L^{-1})
10	MS + NAA (2 mg L^{-1}) + TDZ (2 mg L^{-1})
11	MS + 2,4-D (1 mg L^{-1}) + TDZ (1 mg L^{-1})
12	MS + 2,4-D (2 mg L^{-1}) + TDZ (1 mg L^{-1})
13	MS + 2,4-D (2 mg L^{-1}) + TDZ (2 mg L^{-1})

The MS medium contained 30 g L^{-1} of sucrose with pH=5.6 and 8 g of agar per liter of culture medium. The Petri dishes were covered with parafilm and kept for 16-8 hours (light-dark) at 25°C. As the explants originated from seeds grown in a sterile culture medium, there was no need for sterilization, and no contamination was observed. Additionally, since no phenolic contamination was detected, the use of materials such as activated charcoal was deemed unnecessary. Four explants from one of three explant types were placed in each petri dish and sub-cultured every 4 weeks. After about 80 days, the desired traits were recorded. Trait contained callus formation (%), dry and fresh weight of callus, days to callus formation and mass moisture percentage. The results were analyzed using SAS-9.1 software. The comparison of treatment averages was done based on Duncan's test at the 5% probability level. The calculation of mass moisture percentage was obtained from Equation 1.

$$\text{Mass moisture Percentage} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100 \quad (1)$$

3. Results and discussion

Callogenesis was observed in all treatments except the control. The callus color was different from yellowish white to brown depending on its explants (Fig. 2). Variance analysis results indicate significant differences in all traits based on hormone treatments, explant types, and their interactions (Table 2). Except in control treatment, which lacked hormones and callus was not formed, callus formation was observed in the rest of the treatments (Fig. 2). Hormonal composition and the type of explant and their interaction had very significant effects on days to callus formation, percentage of callus formation, and fresh and dry weight (Table 2). Hormonal composition and the type of explant had very significant effects on mass moisture percentage. However, their interactions have no significant differences (Table 2). The mass moisture percentage was the lowest in NAA (2 mg L^{-1}) + BAP (1 mg L^{-1}) (91.3%) and the highest in 2,4-D (2 mg L^{-1}) + TDZ (2 mg L^{-1}) (95.5%) (Table 3). The highest mass moisture percentage belongs to leaves (87.0%) and the lowest belongs to rootlet (85.7%) explants (Table 4).

Therefore, 2,4-D and TDZ are two hormones that are suitable for maximizing mass moisture percentage in this plant. Thidiazolone (TDZ), a phenylurea derivative, is an effective plant growth regulator with auxin- and cytokinin-like activity in the callus formation process in plants. İşlek (2023) stated that the use of TDZ as a growth stimulant increases the amount of callus formation in cell suspension cultures of *Capsicum annuum* L. compared to the control. In another study, the effect of TDZ on *Artemisia annua* was studied.

The results indicated that the amount of artemisinin in plants regenerated from root explants using 0.1 mg L^{-1} TDZ was determined to be twice more than the control samples (Lualon *et al.*, 2008). One of the uses of TDZ in tissue culture is to stimulate cell division and callus formation; and reduction of callus generation time; The improvement of the morphological and

structural characteristics of calluses was noted. These results align with Ahlawat et al. (2013), who found that maximum callus induction from cotyledon explants occurred in *C. serrata* and *C. senegalensis* on MS medium supplemented with B5 vitamins and 2,4-D (2 mg L^{-1}). Similarly, Mathiyazhagan et al. (2013) reported maximum callus formation with 2,4-D (2.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) in guar genotypes.

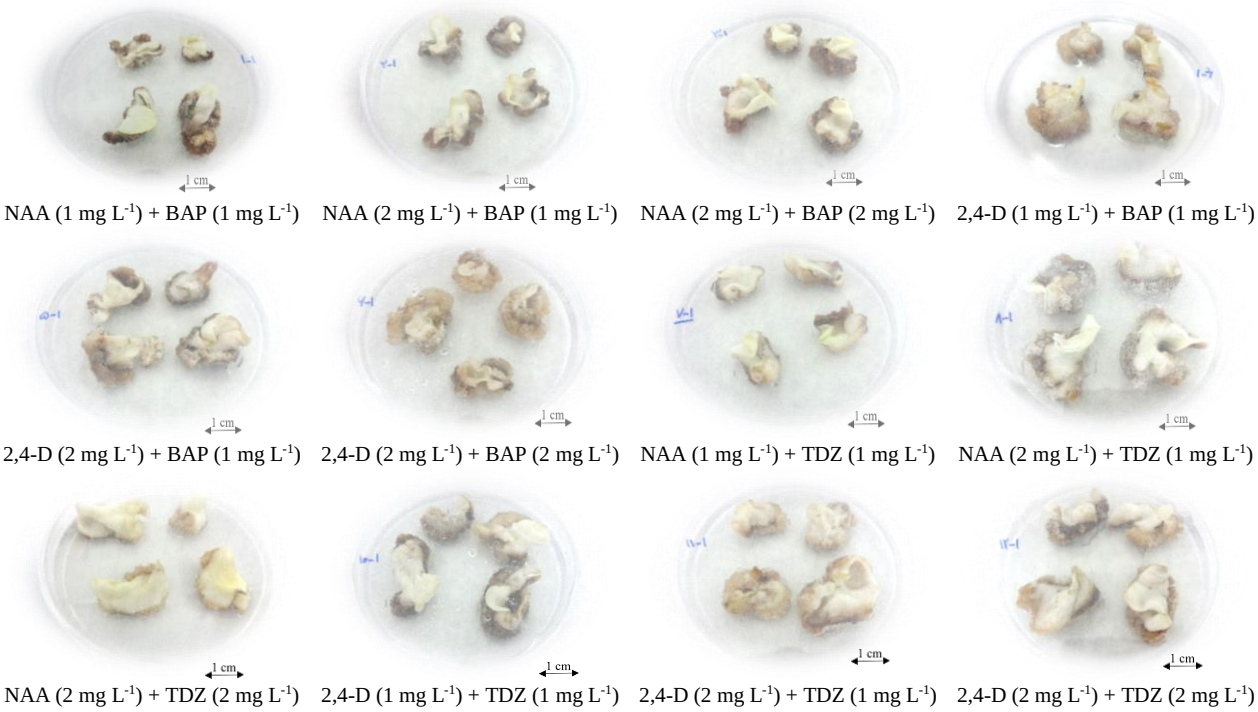

Figure 2. Callus formation in different treatments of hormonal composition in leaf explant after 80 days.

Table 2. Analysis of variance for evaluated traits

S. O. V.	df	Days to callus formation	Callus formation (%)	Fresh weight (g)	Dry weight (g)	Mass moisture percentage (%)
Hormonal composition	12	1814.24**	3.378**	6.0932**	0.0134**	24259.3**
Explant types	2	518.15**	0.341**	68.047**	0.1689**	73.28**
Hormonal composition × Explant types	24	31.82**	0.2782**	1.6644**	0.0032**	10.20ns
Error	429	0.00023	0.0056	0.10561	0.00038	8.0892
C.V. (%)		0.23	0.64	24.3	23.2	3.29

**, *: significant at 1 % and 5 % probability levels, respectively. ns: not significant.

Table 3. Mean comparisons for evaluated traits regarding hormone composition levels

Hormonal composition	Mass moisture percentage (%)
Without Hormones (Control)	0.000^{i}
NAA 1 mg L^{-1} + BAP 1 mg L^{-1}	91.996^{g}
NAA 2 mg L^{-1} + BAP 1 mg L^{-1}	91.358^{h}
NAA 2 mg L^{-1} + BAP 2 mg L^{-1}	92.986^{f}
2,4-D 1 mg L^{-1} + BAP 1 mg L^{-1}	94.418^{cd}
2,4-D 2 mg L^{-1} + BAP 1 mg L^{-1}	94.025^{e}
2,4-D 2 mg L^{-1} + BAP 2 mg L^{-1}	94.585^{bc}
NAA 1 mg L^{-1} + TDZ 1 mg L^{-1}	93.098^{f}
NAA 2 mg L^{-1} + TDZ 1 mg L^{-1}	93.041^{f}
NAA 2 mg L^{-1} + TDZ 2 mg L^{-1}	93.241^{d}
2,4-D 1 mg L^{-1} + TDZ 1 mg L^{-1}	94.603^{b}
2,4-D 2 mg L^{-1} + TDZ 1 mg L^{-1}	94.317^{d}
2,4-D 2 mg L^{-1} + TDZ 2 mg L^{-1}	95.468^{a}

Means with same letter in each column have no significant difference at 5% probability level using Duncan's multiple range test.

Table 4. Mean comparisons of explant types regarding evaluated traits

Explant types	Mass moisture percentage (%)
Leaf	87.032^{a}
Stem	86.203^{b}
Rootlet	85.672^{c}

Means with same letter in each column have no significant difference at 5% probability level using Duncan's multiple range test.

3.1. Interaction between hormonal composition and explant types

According to the analysis of variance, the interaction effects of hormonal compositions and explant-type treatments were very significant in almost all traits (Table 2). In days to callus formation trait, the latest

treatment related to 2,4-D (1 mg L^{-1}) + TDZ (2 mg L^{-1}) in leaf explant (33.0 days), and the earliest treatment related to NAA (2 mg L^{-1}) +TDZ (2 mg L^{-1}) in stem explant (19.0 days) (Table 5). In callus formation percentage, the highest percentage (100%) is related to 2,4-D (2 mg L^{-1}) + TDZ (2 mg L^{-1}) in stem and rootlet explants and in 2,4-D (2 mg L^{-1}) + TDZ (1 mg L^{-1}) in stem explant, and lowest it (20 %) related to NAA (2 mg L^{-1}) + BAP (1 mg L^{-1}) in stem and rootlet explants and NAA (1 mg L^{-1}) + BAP (1 mg L^{-1}) in stem explants (Table 5). In fresh weight of callus traits, the highest value is related to 2,4-D (2 mg L^{-1}) + TDZ (2 mg L^{-1}) in leaf explant and the lowest belongs to NAA (1 mg L^{-1}) + BAP (1 mg L^{-1}) in rootlet explants. Also, in dry weight, the highest value is related to all hormonal composition with TDZ in leaf explants and the lowest belongs to all hormonal composition with BAP in rootlet explants (Table 5). Therefore, TDZ hormone treatment in leaf seedling treatment compared to BAP hormone treatment in root explant treatment produced the highest fresh weight and dry weight. It seems that in this plant, it is better to use leaf explant and TDZ+ 2,4-D hormones treatment to produce calluses with more weight. TDZ and BAP hormones are both cytokinin and play a role in various plant processes including cell growth and proliferation. However, TDZ has stronger cytokinin activity than BAP, which can lead to increased cell division and faster tissue growth. It also has fewer side effects than BAP and can be effective at lower concentrations (Sarwar and Skirvin, 1997).

Table 5. Mean comparison of explant types× hormone composition interactions regarding evaluated traits

Hormones composition × Explant types		Days to callus formation	Callus formation (%)	Fresh weight (g)	Dry weight (g)
NAA 1 mg L^{-1} + BAP 1 mg L^{-1}	Leaf	30.000^{b}	0.600^{f}	1.066^{efghi}	0.077^{cde}
	Stem	25.000^{f}	0.200^{l}	0.530^{kl}	0.041^{ghi}
	Rootlet	25.000^{f}	0.600^{f}	0.207^{l}	0.019^{i}
NAA 2 mg L^{-1} + BAP 1 mg L^{-1}	Leaf	25.000^{f}	0.400^{h}	1.223^{ef}	0.082^{bcd}
	Stem	21.000^{j}	0.200^{l}	0.491^{kl}	0.039^{ghi}
	Rootlet	21.000^{j}	0.200^{l}	0.344^{kl}	0.028^{ghi}
NAA 2 mg L^{-1} + BAP 2 mg L^{-1}	Leaf	27.000^{d}	0.500^{g}	1.787^{cd}	0.108^{ab}
	Stem	20.000^{k}	0.600^{f}	1.147^{efg}	0.074^{cdef}
	Rootlet	22.000^{i}	0.300^{i}	0.281^{kl}	0.022^{i}
2,4-D 1 mg L^{-1} + BAP 1 mg L^{-1}	Leaf	28.000^{c}	0.600^{f}	1.754^{cd}	0.097^{abc}
	Stem	24.000^{g}	0.500^{g}	0.592^{ijkl}	0.033^{ghi}
	Rootlet	22.000^{i}	0.900^{b}	0.537^{kl}	0.030^{ghi}
2,4-D 2 mg L^{-1} + BAP 1 mg L^{-1}	Leaf	22.000^{i}	0.500^{g}	1.815^{cd}	0.109^{ab}
	Stem	25.000^{f}	0.600^{f}	0.546^{jkl}	0.032^{ghi}
	Rootlet	23.000^{h}	0.900^{b}	0.546^{jkl}	0.034^{ghi}
2,4-D 2 mg L^{-1} + BAP 2 mg L^{-1}	Leaf	24.000^{g}	0.800^{c}	2.121^{bc}	0.099^{abc}
	Stem	22.000^{i}	0.900^{b}	0.753^{fghijk}	0.040^{ghi}
	Rootlet	23.000^{h}	0.900^{b}	0.666^{hijkl}	0.042^{ghi}
NAA 1 mg L^{-1} + TDZ 1 mg L^{-1}	Leaf	26.000^{e}	0.300^{i}	1.465^{de}	0.090^{abc}
	Stem	25.000^{f}	0.700^{d}	1.090^{efgh}	0.071^{cdef}
	Rootlet	26.000^{e}	0.300^{i}	0.311^{kl}	0.024^{hi}
NAA 2 mg L^{-1} + TDZ 1 mg L^{-1}	Leaf	25.000^{f}	0.600^{f}	2.298^{b}	0.115^{a}
	Stem	20.000^{k}	0.800^{c}	0.627^{hijkl}	0.052^{efgh}
	Rootlet	21.000^{j}	0.700^{d}	0.594^{ijkl}	0.048^{fghi}
NAA 2 mg L^{-1} + TDZ 2 mg L^{-1}	Leaf	24.000^{g}	0.299^{j}	1.420^{de}	0.089^{abc}
	Stem	19.000^{l}	0.690^{e}	1.100^{efgh}	0.073^{cdef}
	Rootlet	22.000^{i}	0.295^{k}	0.321^{kl}	0.026^{hi}
2,4-D 1 mg L^{-1} + TDZ 1 mg L^{-1}	Leaf	33.000^{a}	0.700^{d}	2.178^{bc}	0.111^{a}
	Stem	28.000^{c}	0.900^{b}	0.754^{fghijk}	0.038^{ghi}
	Rootlet	27.000^{d}	0.800^{c}	0.435^{kl}	0.026^{hi}
2,4-D 2 mg L^{-1} + TDZ 1 mg L^{-1}	Leaf	28.000^{c}	0.700^{d}	2.340^{b}	0.113^{a}
	Stem	26.000^{e}	1.000^{a}	1.030^{efghij}	0.057^{defg}
	Rootlet	26.000^{e}	0.900^{b}	0.439^{kl}	0.029^{ghi}
2,4-D 2 mg L^{-1} + TDZ 2 mg L^{-1}	Leaf	27.000^{d}	0.700^{d}	2.786^{a}	0.117^{a}
	Stem	22.000^{i}	1.000^{a}	0.676^{ghijkl}	0.032^{ghi}
	Rootlet	21.000^{j}	1.000^{a}	0.610^{ijkl}	0.027^{ghi}

Means with same letter in each column have not significant difference at 5% probability level using Duncan's multiple range test.

According to these results, the MS media with 2,4-D and TDZ, could lead to more callus formation with more mass moisture percentage of the guar. Since the most significant callus formation was observed with the MS medium supplemented with 2 mg L^{-1} of both 2,4-D and TDZ, exploring different combinations of these hormones could be beneficial. Better results might be achieved by increasing their concentrations. In addition to the hormones used in this research, the effect of other plant growth hormones can also be investigated. Some hormones may have different effects on callus formation. Also, temperature, humidity, light and other physical factors can have a different effect on callus formation. Therefore, it is suggested to investigate and optimize the effect of these factors to choose the best conditions for callus formation.

3.2. Correlation between traits

The results of the Pearson's correlation coefficients show that there is a positive and significant correlation between the days to callus formation and dry weight ($r=0.425^{**}$) and fresh weight ($r=0.416^{**}$), but no significant correlation was observed between these traits and the mass percentage (Table 6). The reason for this correlation between the days to callus formation and callus weight can be related to the fact that by increasing the time required for callus formation, the cells have more time to increase their dry weight and fresh weight. These results are consistent with Rathod et al. (2017) in soybean callus formation research. A significant correlation was observed between the percentage of callus formation and fresh weight ($r=0.325^{**}$) and mass moisture percentage ($r=0.513^{**}$), but this trait did not have a significant correlation with dry weight. A significant correlation was observed between fresh weight and dry weight ($r=0.955^{**}$). The correlation of these two traits with mass moisture percentage was significant (Table 6).

Table 6. Correlation coefficients between evaluated traits

	Days to callus formation	Callus formation (%)	Fresh weight (g)	Dry weight (g)
Callus formation (%)	0.503^{**}			
Fresh weight (g)	0.416^{**}	0.325^{**}		
Dry weight (g)	0.425^{**}	0.265^{**}	0.955^{**}	
Mass moisture percent (%)	0.053^{ns}	0.513^{**}	0.349^{**}	0.321^{**}

**, *: significant at 1% and 5% probability levels, respectively. ns: not significant.

4. Conclusion

This study showed that treatment of guar with 2 mg L^{-1} TDZ and 2 mg L^{-1} 2,4-D significantly increased the rate of callus formation and its weight, especially in leaf explants. Also, The results showed that although both TDZ and IBA are cytokinin hormones, TDZ can be more effective in callus formation and weight when combined with 2,4-D. TDZ works better than IBA because it increases the rate of cell division and reduces adverse side effects. This indicates that 2,4-D and TDZ are effective hormones for optimizing callus development in this plant. These findings highlight the presence of an efficient method for generating calli in guar, offering high-performing cells useful for exploring physiological and molecular processes. Changing the environmental conditions during cultivation, such as adjusting the day length along with light and temperature settings, may enhance callus formation in this plant.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this research.

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