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RESEARCH ARTICLES

# Development of an efficient *in vitro* protocol to increase callus induction and regeneration rate in soybeans (*Glycine max* L. Merrill)

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## Abstract

Soybean is a legume crop that is highly dependent on various parameters and is difficult to maintain in vitro. Tissue culture techniques have been optimized to develop a standardized methodology for soybean. The most significant constraints that impede the efficacy of current techniques in soybean tissue culture studies are commonly dependent on the genotype, explant type, and media composition. In this study, the frequency of callus formation and the regeneration capacity of three different cultivars, Arısoy, Nova and Blaze, that had not previously been investigated in vitro were evaluated for various media contents, CIM1 (1 mg $L^{-1}$ 2,4-D+0.1 mg $L^{-1}$ Kinetin), CIM2 (2 mg $L^{-1}$ BAP+2 mg $L^{-1}$ NAA) and CIM3 (1 mg $L^{-1}$ BAP+0.1 mg $L^{-1}$ NAA+0.1 mg $L^{-1}$ GA3+0.1 mg $L^{-1}$ TDZ ), and explant types, cotyledonary leaves, cotyledonary node, embryo and hypocotyl. The highest calli induction for Arısoy, Nova, and Blaze cultivars was obtained at CIM1 (70%), CIM2 (56%), and CIM3 (56%) in terms of media composition, and in hypocotyl (98%) and embryo (66% and 65%) as explant types. The interactions of all CIM × hypocotyl (98%) and CIM1 × cotyledonary node (100%) for Arısoy, and CIM1 × embryos (91%) and CIM2 × embryos (100%) for Nova and Blaze, respectively, had the greatest rate of calli production. Arısoy was determined to be the best cultivar for callus response based on all treatments. The calli of both Arısoy and Nova cultivars achieved a regeneration rate of 22% and 14%, respectively, when transferred to regeneration medium containing 0.45 µM TDZ, however with only cotyledonary nodes showing shoot initiation and elongation. As these cultivars have not previously been studied in vitro in terms of callus response and regeneration, it is expected that they will play an important role in genetic transformation studies in the future.

**Keywords** Soybean · Callus · Regeneration · Cotyledonary node · Hypocotyl

## Introduction

Soybean is an important oil crop and a member of the legume family. It is very rich in protein (35–45%), oil, carbohydrates, fiber, and vitamin content (Sharma et al. 2014). This crop serves as a staple food source (i.e., tofu, soy milk, soy nut, and edamame), particularly in East Asia. Scientists are working on creating biofortified crops via different strategies, but soybean have gained strategic importance already due to its inherent rich nutritional value (Croser et al. 2006). It has the potential to address hunger in the least developed and developing nations, and can be seen as a substitute for groundnut and cowpea cultivation in the region for the future (Raza et al. 2017; Keatinge et al. 2011; Allen and Boote 2000). Soybean production is gradually rising globally, in line with the expansion of cultivation areas and yield improvements after intensive breeding efforts (FAO 2023).

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Soybean is one of the most recalcitrant legume crops to handle and maintain in tissue culture (Stephens et al. 1991). A reproducible, standardized, and optimized tissue culture system for soybean is yet to be developed, as the success of the process depends heavily on various factors, such as genotype, media composition, growth regulator, explant type and size, photoperiod, temperature, seed age, and soybean maturity group (Reichert et al. 2003; Tiwari et al. 2011; Raza et al. 2020). Several particular cultivars, including Maverick, Williams 82, and Jack, have been discovered to be highly responsive to calli induction/shoot regeneration and organogenesis after extensive studies on optimizing the most responsive soybean genotypes in vitro (Christou and Swain 1990; Samoylov et al. 1998; Cui et al. 2013; Seo et al. 2021). Studies have shown that a variety of explant sources are frequently used for in vitro propagation and genetic transformation and are adjusted for further in vitro purposes in soybean, including half seeds (Paz et al. 2006), young seedlings (Atak et al. 2007), hypocotyl (half-split and complete) (Dan and Reichert 1998; Raza et al. 2017), (whole) cotyledonary nodes (Paz et al. 2004; Olhoft et al. 2006), cotyledonary leaves (Faraz et al. 2019), immature cotyledons (Hofmann et al. 2004), and embryonic axis (Cho et al. 2022). The success rate of callus initiation and formation in soybean is quite high (>90%), but studies have reported very low regeneration success rates (Tripathi et al. 2004). Regeneration of shoot from calli has only been reported in cotyledonary node explants, with a low rate of regeneration observed to date (Sairam et al. 2003; Ma and Wu 2008). One main disadvantage of using cotyledonary nodes in plant transformation during transgenic studies is the high chance of getting chimeric plants (Meurer et al. 1998). Although a study reported successful regeneration of calli developed from leaf, shoot, and root explants using varying 2,4-D and kinetin concentrations calculated by a statistical modelling, no success has been achieved for other explant types in soybean, except for the cotyledonary node. Unfortunately, this is the only study to demonstrate the possibility of shoot regeneration of these explants (Abbasi et al. 2016; Raza et al. 2017). Various parameters, including media composition, growth regulators, and genotypes, have been tested for different explant types. However, it has been determined that this issue is likely to be associated with poor totipotency in soybean after germination (Saka et al. 1980; Olhoft et al. 2003).

*In vitro* soybean culture techniques are typically classified into methods based on somatic embryogenesis and organogenesis. The most typical explant sources for both approaches are the cotyledonary nodes (Sairam et al. 2003; Li et al. 2017). Although it has only been shown in a small number of studies that using variouos carbon sources could enhance genotype-independent regeneration in cotyledonary node explants, the success is still highly dependent on genotype. However, these research are still constrained (Sairam et al. 2003). This study aimed to test the response of several factors, including four different explant types, three different media contents, and three different genotypes, to observe callus initiation, development, and regeneration frequency for local soybean varieties in Türkiye.

## Materials and methods

### Plant Material and in Vitro Germination

Initially, mature seeds of eight cultivars (Arısoy, Nova, Blaze, SA-88, Atakişi, Maverick, Umut 2002, and Bravo) from two different maturity groups (III and IV) with various hilum colors (brown, dark brown, black, red, and yellow) were used in the study to assess their germination rates (Çalışkan and Aytekin 2017).

Germination Rate (%): (Germinated Seed / Total Seed)*100.

Mature soybean seeds underwent surface sterilization with 70% ethanol (Tekkim) for 4 min and were subsequently placed in a 1% bleach with Tween 20 (PanReac AppliChem) in for 8 min and sterilized with constant shaking in a falcon tube. Afterward, the seeds were washed with sterile water and completely dried on sterile tissue paper in a sterile cabinet for about 10 min. Sterilized seeds were germinated in a Magenta box with germination medium (~100 ml culture medium) (4.4. g $L^{-1}$ MS salts with vitamins, 8 g $L^{-1}$ plant agar, and 30 g $L^{-1}$ sucrose; pH:5.7) (Duchefa Biochemie) under a 16/8 h photoperiod at 25°C. The germination experiment was planned to have three replications, each with twelve seeds and the experiment was performed twice. 10–15 days old germinated seedlings were used as main explant sources for cotyledonary node, cotyledonary leaves and hypocotyls. The study was performed using three cultivars, Blaze, Nova, and Arısoy, which had the highest germination rates (>80%) for callus induction and regeneration.

### Callus induction and shoot regeneration

Four explant types (cotyledonary node, cotyledonary leaves, hypocotyls, and embryos) and three local cultivars (Blaze, Nova, and Arısoy) were included in this study. Hypocotyls of approximately 1 cm in size was used for callus induction. First, the cotyledonary leaves were cut from the sides, as similar to a rectangular shape, and then the remaining cotyledon node part was placed in an adaxial position to the media. A seperate experiment was conducted for the last explant type, embryo, in this study. Surface-sterilized mature seeds were kept in sterile water and in the dark at

**Table 1** Callus induction and growth, and regeneration media composition, and concentration of growth regulators

| Name | Growth Regulator | MS Basal Media (1 L) |
|---|---|---|
| CIM1 | 1 mg $L^{-1}$ 2,4-D + 0.1 mg $L^{-1}$ Kinetin | 4.4 g MS 0.8% agar 3% sucrose pH: 5.7 |
| CIM2 | 2 mg $L^{-1}$ BAP + 2 mg $L^{-1}$ NAA | |
| CIM3 | 1 mg $L^{-1}$ BAP + 0.1 mg $L^{-1}$ NAA + 0.1 mg $L^{-1}$ $GA_3$ + 0.1 mg $L^{-1}$ TDZ | |
| RIM | 0.45 µM TDZ | |

Note 1: CIM: callus induction media, RIM: regeneration induction media

Note 2: 2,4-D: 2,4-D Dichlorophenoxy acetic acid, BAP: 6-Benzylaminopurine, NAA: 1-Naphthaleneacetic acid, $GA_3$: Gibberellic acid, TDZ: Thidiazuron

**Table 2** The detailed information about soybean cultivars and their in vitro germination rates ($p < 0.05$, Tukey's test)

| Cultivar | Origin | Maturity Group | Germination percentage (%) |
|---|---|---|---|
| SA-88 | Türkiye | III | 75 ± 36.3a |
| Atakişi | Türkiye | III | 56 ± 4.8ab |
| Blaze | Türkiye | IV | 94 ± 4.8a |
| Nova | Türkiye | III | 92 ± 14.4a |
| Arısoy | Türkiye | III | 94 ± 4.8a |
| Maverick | USA | III | 56 ± 17.3ab |
| Umut 2002 | Türkiye | IV | 22 ± 9.6bc |
| Bravo | Türkiye | III | 0 |

low temperature (4°C) for two days. The embryos were then carefully removed from imbibed seeds without any damage by removing the seed coat under sterile conditions and incubated for callus induction. The experiment was set up in a completely randomized design with three replications. Each replicate comprised one glass petri dish (each with ~40 ml culture medium) and six explants. Three different media compositions, CIM1, CIM2, and CIM3, with various growth regulators and concentrations, were used to trigger callus initiation and growth in the study (Table 1). All growth regulators used in the study were obtained from Duchefa Biochemie.

The explants were monitored daily for callus formation in order to calculate both days to callus initiation and % callus induction. The pictures of calli were obtained using steromicroscope (Leica EZ4). Calli were transferred to the regeneration media provided in Table 1 to promote shoot regeneration from callus-forming explants (Hong et al. 2007). Sub-culture was performed every month in regeneration medium, and the medium was refreshed to avoid browning and tissue necrosis. In the regeneration medium, calli were maintained under the same photoperiod and temperature conditions as in the callus induction and growth medium. The number of regenerated calli was divided by the total number of explants and multiplied by 100 to calculate regeneration frequency. ANOVA test for mean comparison and post-hoc test (Tukey's test) were conducted using SAS Software, and statistical significance was indicated by a p value less than 0.05.

## Results

Seeds were germinated in MS medium to calculate the in *vitro* germination frequencies of the eight soybean cultivars, six of which were from maturity group III, and the rest were from maturity group IV (Table 2). All cultivars, except Maverick, were local cultivars developed in Türkiye. Blaze, Nova, and Arısoy cultivars had the highest germination rates (>90%) (Table 2). The germination rates of the rest were as follows: SA-88 (75%), Atakişi (56%), Maverick (56%), and Umut 2002 (22%). Cultivar Bravo has not germinated at all. The three cultivars with the highest germination rates, Blaze, Nova, and Arısoy, were used in further studies. The mean days to germination (green cotyledons with elongated hypocotyl stage) varied between 5 and 8 days, Arısoy the earliest (approx. 5-day) and Blaze the latest (approx. 8-day).

Callus formation was initiated with explants, hypocotyls, cotyledonary nodes, and leaves using 10–15 old seedlings, and the seeds were kept in sterile water for excision of embryo explants. The explants from three different cultivars were transferred to three different CIM with different growth regulator compositions and rates, the details of which are given in Table 1. The individual factors (genotype, explant, and media type) and their interactions (explant × genotype, explant × media, genotype × explant × media) were significant ($p < 0.01$) according to the ANOVA findings. However, the media content × genotype interaction did not appear to be significant.

Hypocotyls and CIM1, which produced 100% and 70% calli, respectively, displayed the highest callus induction rates in terms of explant type and media composition in cv. Arısoy (Fig. 1). Calli obtained from various explants in cv. Arısoy is depicted in Fig. 2. The lowest callus induction rates were observed in cotyledonary leaves (18%) and CIM3 (42%). Five days after transfer to CIM, calli initiation and induction began in all explant types, unlike in cotyledonary leaves (13-day in CIM3 and 35-day in CIM2). The hypocotyl and embryo produced large calli and had high callus quality in all CIMs. Although the rate of callus generation in CIM1 was higher, the callus quality was observed to be better in CIM2. While the calli derived from cotyledonary nodes were in a non-friable form, those derived from cotyledonary leaves, the hypocotyl, and the embryo were soft, watery and friable in Arısoy. The callus color was typically green, although healthy yellow callus formation was occasionally observed in some explants, such as cotyledonary nodes and leaves.

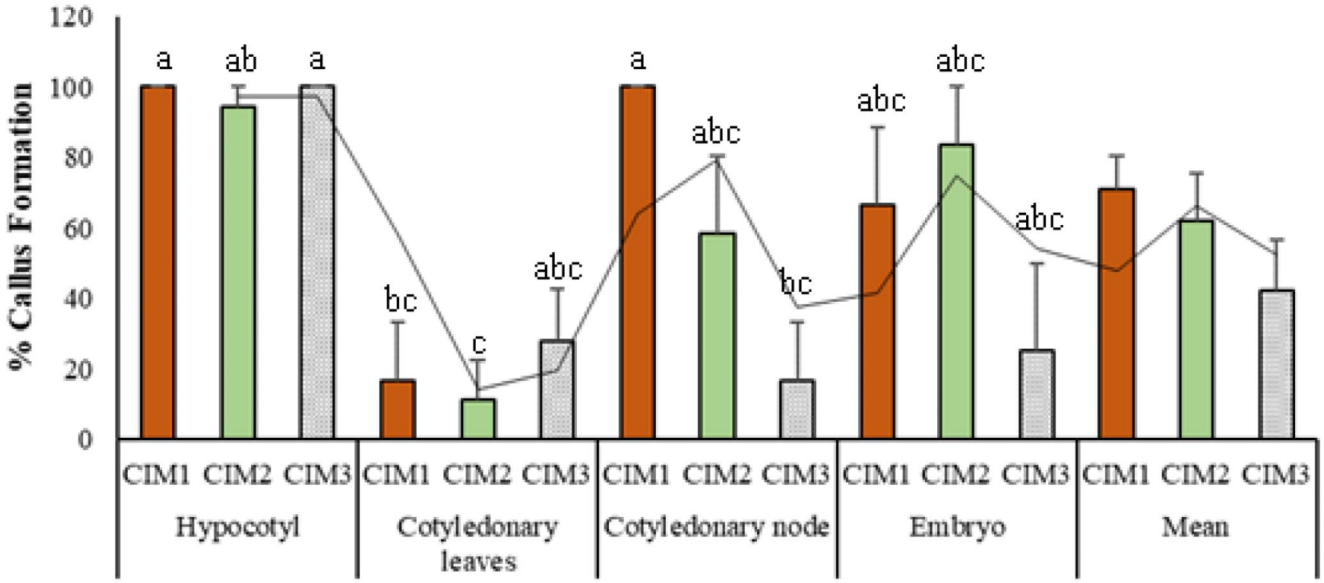


**Fig. 1** Responses of different explant types to different media contents in cv. Arısoy ($p < 0.05$, Tukey's test)

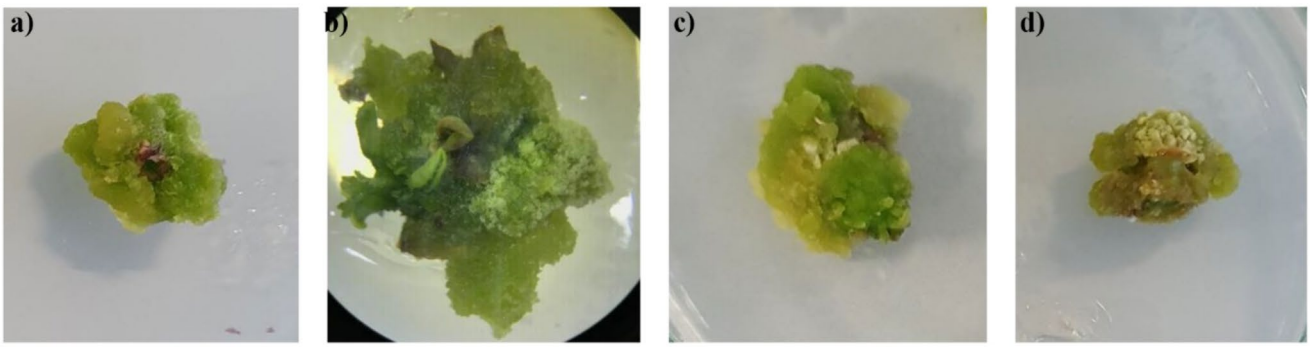


**Fig. 2** Calli formation in cv. Arısoy from different explant types, **(a)** cotyledonary leaves, **(b)** cotyledonary node, **(c)** embryo, and **(d)** hypocotyl

The hypocotyl and embryo showed the highest callus development rates, with CIM1-72% and CIM2-88% for the hypocotyl and CIM1-91% for embryos in cv. Nova (Fig. 3). Although the media content × genotype interaction was not statistically significant, the findings showed that CIM2 had the highest callus formation rate (56%), followed by CIM1 (43%) and CIM3 (16%). There was no callus formation on cotyledonary leaves for CIM1 and CIM3. The explants that formed the lowest callus rates were cotyledonary leaves (20%) and nodes (25%) for cv. Nova. The mean time range for callus development varied from 5-day (CIM2-cotyledonary node and CIM2-hypocotyl) to 34-day (CIM1-cotyledonary node and CIM3-embryo), and the days to callus induction for other media × explant interactions ranged between 13 and 21 days. While the cotyledonary node produced non-friable calli, calli developed from the cotyledonary leaves, hypocotyls, and embryos were friable. All cotyledonary leaves showed necrosis before callus initiation, although necrosis began after callus development in the CIM2. While almost all calli developed green tissues, interestingly, the embryos formed friable white callus in CIM2 for cv. Nova. The callus quality was poor in CIM1 and CIM3. Embryo produced the highest quality calli in general for cv. Nova.

The highest callus production rate for cv. Blaze was measured in the embryo at 63%, whereas the lowest rate was found in the hypocotyl at 18%, unlike that in Arısoy and Nova (Fig. 4). Callus formation rates were 31% and 19% for the cotyledonary leaves and nodes, respectively. CIM2 showed a greater mean callus production (56%) than CIM1 (21%) and CIM3 (23%). Callus initiation and growth were not observed in the cotyledonary leaves of CIM1 or cotyledonary nodes of both CIM1 and CIM3. Initial callus formation was noticed five to twenty-one days after transferring explants to the media for cv. Blaze. Other explants and media types, however, had an average callus development time of thirteen days. The cotyledon node and embryo were identified as the two explants with the best callus qualities.

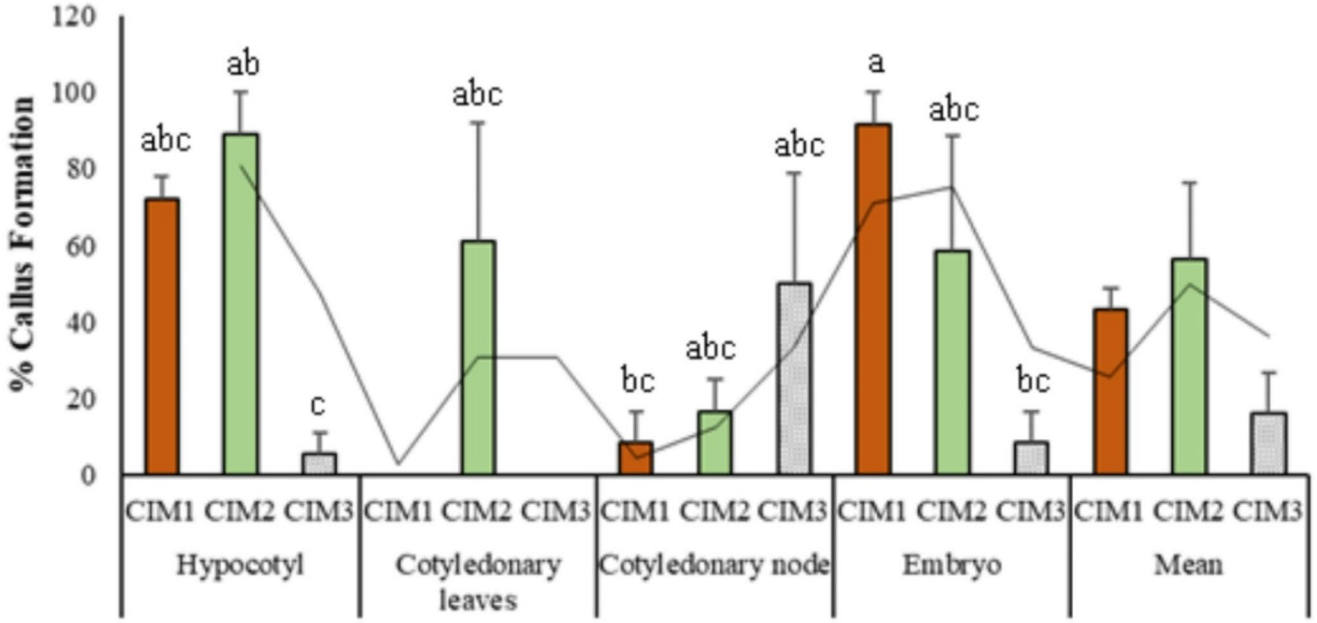


**Fig. 3** Responses of different explant types to different media contents in cv. Nova ($p < 0.05$, Tukey's test)

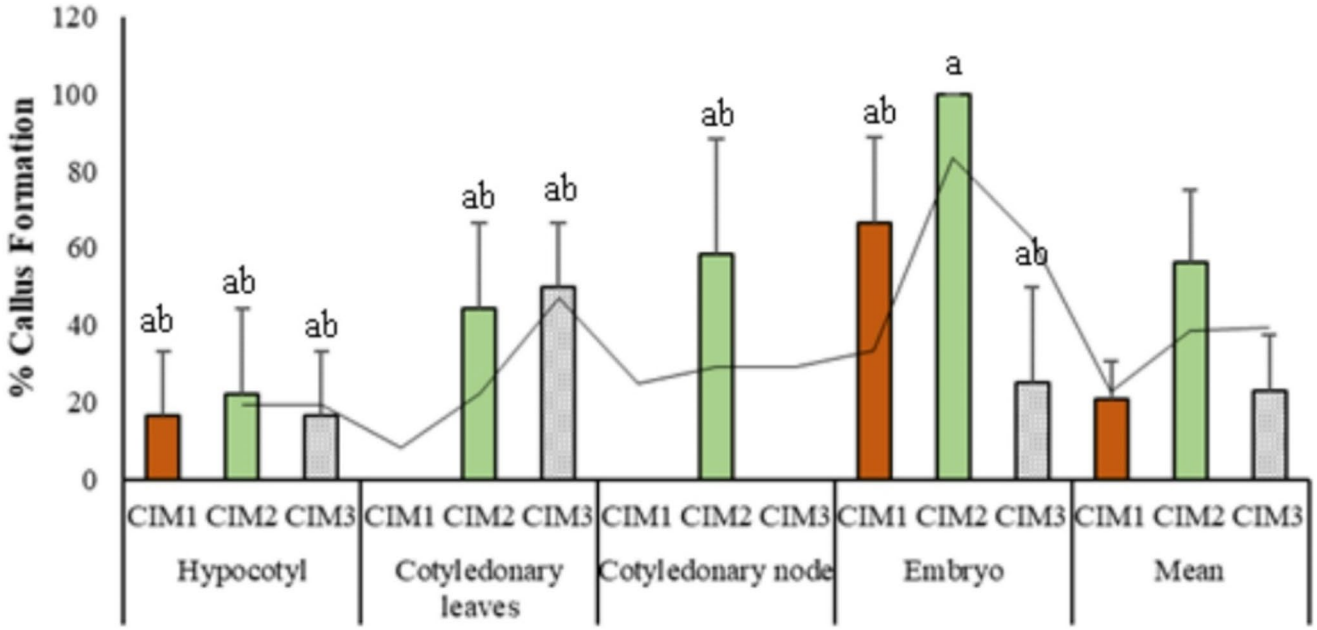


**Fig. 4** Responses of different explant types to different media contents in cv. Blaze ($p < 0.05$, Tukey's test)

Blaze cultivar was observed to be less effective in producing calluses compared to the Arısoy and Nova cultivars. CIM3 produced friable and green calli in all explant types, whereas CIM1 showed necrosis after callus initiation. The embryos mostly formed white calli in cv. Blaze. Since the shoots of the Blaze cultivar were purple, the necrosis rate in the tissues was higher than that in other experiments probably due to the toxic effect of phenolic accumulation.

The calli were transferred to TDZ medium containing 0.45 µM for regeneration into shoot. Regeneration was only observed in calli developed using cotyledonary nodes from Arısoy and Nova; however, regeneration took an average of two–three months. No explant type was regenerated for cv. Blaze. The shoots in Arısoy had just begun to form, while the shoots in Nova started to elongate (Fig. 5). The leaves of regenerated plants, particularly in Nova, mostly turned yellow in color. The regeneration rate was determined to


**Fig. 5** The regeneration (in 0.45 µM TDZ) from cotyledonary nodes **(a)** in cv. Arısoy and **(b)** cv. Nova

be 22% in Arısoy and 14% in Nova. Regeneration was observed only in the cotyledonary node; calli from cotyledonary leaves, embryos, and hypocotyls did not form any shoots.

## Discussion

Maintenance and handling of soybeans in tissue culture is difficult, especially because of the lack of regeneration and reliance on the genotype and explant type for the induction of callus and shoot regeneration. The callus formation frequency and regeneration capacity of three different cultivars, which have not been previously studied in vitro, were tested for different media contents and explant types in this study.

Arısoy was the most responsive cultivar to callus induction among the three cultivars studied, and demonstrated the ability to form calli for all explant types and under all media compositions. There was no indication of callus growth in Nova (CIM1 and CIM3) or Blaze (CIM1) for cotydonary leaves and CIM1 and CIM3 for cotyledonary nodes. CIM1 and CIM3 demonstrated the highest degree of callus production in Arısoy and Nova, respectively. The hypocotyl was the most responsive explant type for callus formation for latter cultivars. In contrast, Blaze exhibited significantly decreased callus formation in the hypocotyl. The purple pigmentation of the Blaze may have hindered callus development despite the use of vitamin-containing MS, which is known to reduce necrosis-caused tissue darkening and discoloration (Phat et al. 2015).

Auxin, cytokinin, and their combinations have been successfully utilized to induce callogenesis in soybean. Callus formation in cotyledons and embryos was previously reported in studies using auxin, 2, 4-D, NAA, and 2,4-D+NAA (Joyner et al. 2010; Islam et al. 2017). The callus formation rate increased from 54 to 67% in the three different varieties when compared to the treatments in which auxin was applied alone and in combination with auxin and cytokinin (2,4-D and BAP at different rates), respectively (Islam et al. 2017). In another study, callus growth was observed to be promoted in both shoots and leaves at the same concentrations of 2, 4-D and kinetin, as well as when 2,4-D was at concentrations five times that of kinetin (Abbasi et al. 2016). The combination of NAA and kinetin increased callus formation in hypocotyls (Liu et al. 1997). The application of BAP and GA3 at a ratio of 1:0.25 led to a notable increase in callus development in the cotyledonary node in Korean soybean cultivars (Phat et al. 2005). Although TDZ is typically added to soybean medium for regeneration purposes, it has been demonstrated that a combination of 2,4-D and TDZ can also lead to the development of calli. Despite investigating different concentrations in the latter study, the average time for callus formation was found to range from 25 to 35 days, as reported by Kumar et al. (2021). In our research, we added TDZ to CIM3 to observe the effect on the formation of calluses, and found that the average time required for callus formation was between 5 and 34 days. Notably, spontaneous regeneration did not occur during callus development in our study. Although the genotype × media interaction was not statistically significant, the explant × media and genotype × explant × media interactions were significant in our study. Therefore, in our study, different combinations of auxin and cytokinin were used to successfully induce callus development, which is in line with prior findings where the range for successful callus induction and regeneration in soybean tissue culture are generally considered to be 40% and 5–40%, respectively (Islam et al. 2017; Hada et al. 2018; Paz et al. 2004).

Regeneration, including shoot initiation and elongation, was only observed in the cotyledonary nodes in our study. Our results are consistent with those of previous studies, which did not report any regeneration in cotyledonary leaves, hypocotyls, or embryos, except for spontaneous adventitious regeneration in the hypocotyl explant and embryonic axes in one study. Somatic embryos developed from the hypocotyl can germinate and trigger adventitious shoot initiation; however, their reproducibility and implementation are limited in soybean (Ghazi et al. 1986; Dan and Reichert 1998). Previous research has indicated that

primary leaves, as well as cotyledonary leaves, are not efficacious in shoot regeneration. While primary leaves of different sizes and 2.4-T in media have been shown to stimulate regeneration at rates from 0 to 50%, root elongation has been found to be delayed in regenerated plants (Wright et al. 1987). In a further study, it was found that leaf discs were effective for shoot induction when the BAP was low and the auxin levels were high (Tripathi and Tiwari 2004).

Excision of embryonic axes from mature soybean seeds has been demonstrated to be effective in stimulating regeneration in various studies and has been shown to be competent in soybean shoot initiation and elongation, unlike our study (Tripathi and Tiwari 2004). However, this explant type is dependent on the genotype and reproducibility is low (Komatsuda et al. 1991). The explant type that is most appropriate for the regeneration of soybean calli in tissue culture and for genetic transformation studies has been identified as the cotyledonary node. This was confirmed by the results of our study on Arısoy and Nova.

## Conclusion

Today, many plants, including soybean, require reliable systems that are optimized, especially for genetic transformation studies, and have a high success rate, where high callus formation and regeneration can be repeated regardless of factors such as genotype, explant, and media content. There is still no protocol that optimizes the conditions in soybean so that for each cultivar planned to be studied, the most ideal conditions must be met by testing some important parameters such as explant type, media content, and growth regulator. For this reason, in this study, different factors were investigated in different cultivars and the optimal conditions were adapted, and important results were obtained in terms of both callus formation and regeneration. The results of this study will make great contributions to in vitro and genetic transformation studies in soybean.

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**Author contributions** CY, AB, and MEÇ conceived and designed the research. CY, BAD, MT conducted all experiments. CY and MEÇ analyzed data. CY wrote the manuscript. CY, BAD, MT, AB, and MEÇ edited the manuscript. All authors read and approved the manuscript.

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## Declarations

**Consent for publication** Authors do have consent for publication.

**Competing interests** The authors declare that they have no conflict of interest.

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