*Pol. J. Environ. Stud. Vol. 33, No. 2 (2024), 1421-1427*
DOI: *10.15244/pjoes/173443* ONLINE PUBLICATION DATE: 2023-11-24

*Original Research*

# The Effect of $H_3BO_3$ and 2,4-D on *in vitro* Callus and Somatic Embryo Formation of Bitter Melon (*Momordica charantia* L.)

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*Received: 19 July 2023*
*Accepted: 6 October 2023*

## Abstract

Bitter melon (*Momordica charantia* L.) is an economically important medicinal and vegetable crop plant that is rarely grown in Turkey under natural conditions. This study aimed to develop an *in vitro* somatic embryo regeneration method using dissimilar concentrations of $H_3BO_3$ and 2,4-D on leaf and stem explants of the Gazipasa bitter melon ecotype. Boron is an essential microelement for plants, and its deficiency or excess creates significant problems in plant development. Within the scope of the study, 0.5, 1.0, 1.5, and 2.0 mg $l^{-1}$ concentrations of $H_3BO_3$ and 1.0 and 2.0 mg $l^{-1}$ 2,4-D were applied to the leaf and stem explants. The results showed 100.00% and 60.00% callus formation on leaf explants after two weeks and stem explants after 3 weeks using MS medium amended with 0.5 mg $l^{-1}$ $H_3BO_3$ and 2.0 mg $l^{-1}$ 2,4-D. Somatic embryo formation percentages were dependent on the type of explant. Their recovery to healthy plantlets was obtained on MS medium. The somatic embryos at the globular and heart stages comprised identifiable protoderm layer formation that was not observed if $H_3BO_3$ was not added into the cultures and the embryos were fused with hyperhydricity. The study meets the objectives of the research.

**Keywords**: $H_3BO_3$, *in vitro* somatic embryogenesis, callus formation, bitter melon, 2,4-D

## Introduction

The genus *Momordica* consists of 59 species, of which about ten are distributed in Africa and Southeast Asia [1-3]. *Momordica charantia* L. Cucurbitaceae family (Bitter melon syn. karela, bitter melon, bitter cucumber, balsam apple, balsam pear, bitter gourd, fu kwa, nigai uri, ampalaya, karela, papailla, pavaaki, salsamino, peria, sorossies, chin li chih and goo-fash) is economically important medicinal and vegetable plant [4]. It is a climbing and annual herb that is widely cultivated in South Asian countries (Pakistan, Afghanistan, India, Nepal, Bangladesh, Bhutan, Sri Lanka), Russia, the Southern part of the Arabian Peninsula (Yemen), some African, and Central European countries [5]. It is very rarely cultivated as a vegetable

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but grows under natural conditions for use in various medicinal formulations of the ethnomedicinal system of the Aegean region of Turkey [6].

Many plant species in this family are used in ethnopharmacology in many cultures, countries and regions [7]. *M. charantia* fruits and leaves have been applied externally as anthelmintic in the treatment of leprosy [8]. It has been used as an antipyretic to treat rheumatism, gout, liver, and spleen diseases [9]. The ripe fruits are used externally to heal wounds and in the treatment of stomach ailments including oral peptic ulcers in Turkish folk medicine [10]. It is part of the traditional diet of the inhabitants of Pakistan, some parts of India, and Japanese Okinawa Island [11]. It is traditionally used in the treatment of diabetes in China [12]. Bioactive components of *M. charantia* act enhancing tumor suppressor gene activity [13]. Its fruits are rich in iron, calcium, and phosphorus, containing substances with antidiabetic properties such as charantin, vicin, polypeptide-P, and other non-specific bioactive components such as antioxidants [14]. The bitterness of this plant is attributed to its saponins, and mainly four cucurbit glycosides namely momordicines I, momordicines II, momordicosides K and momordicosides L. [15, 16].

Tissue culture is the production of plants and plant products in an artificial nutrient medium under aseptic conditions involving basic genetic and biochemistry research in cell biology as well as commercial applications including tissue culture, genetic transformation, and editing [17]. These studies are carried out by using cell cultures, anthers, ovules and embryos, protoplast isolation, their fusion, appropriate cell selection, bud cultures, and callus and suspension cultures [18]. Clonal propagation, production of haploid plants and breeding studies to overcome incompatibility problems [19, 20].

Recent developments in genetic engineering are very important in the production and reproduction of these plants. Many bioengineering applications in the biotechnology of plants have been studied intensively in the last forty to fifty years. Callus cultures and suspension cell cultures offer a wide range of uses in pharmacology and pharmacy as well as in other branches of agriculture [21-23]. Genetically modified callus cultures developed through gene technologies can be used to produce plants with increased resistance to salt, drought, diseases, and pests by the synthesis of bioactive secondary metabolites [24]. Although the full potential of callus plant culture technology has not yet been exploited, the time has come to develop and market more callus culture-based products [25].

This study, aimed to investigate the effects of different $H_3BO_3$ and 2,4-D concentrations on somatic embryo formation of leaf and stem explants of bitter gourd.

## Material and Methods

### Plant Material

The seeds of the bitter gourd plant were obtained from the Gazipasa county of Antalya province in 2020 (Fig. 1A). The seeds were sown directly into pots containing peat:perlite (1:1) mixture at a depth of 1.0 cm to multiply them. Once the vines were established, they were identified at the flowering and fruit setting stage by the author [26] using the keys as given in the flora of China [27] and Flora of Pakistan [28].

### Surface Sterilisation and Germination

The fresh seeds from these plants were surface sterilized under aseptic conditions using 10% NaOCl (0.5% commercial bleach sold as Ace, Istanbul Turkey) under *in vitro* conditions, followed by rinsing with bidistillled water for 3 min × 6 times in a biosafety cabinet. Thereafter, the seeds were germinated under aseptic conditions using sterilized wet cotton. The leaf and stem explants were aseptically cut using sterilized scalpel blades No. 10 and forceps from 6-7 days old germinated plants in the petri dishes containing respective concentrations of boric acid and 2,4-D amended MS culture medium [29].

### Boron Salt ($H_3BO_3$) and 2,4-D Treatments

These explants were treated with four different concentrations (0.5, 1.0, 1.5, 2.0 mg $l^{-1}$) of $H_3BO_3$ (Boric acid) and two different concentrations (1.0, 2.0 mg $l^{-1}$) of 2,4-D (2,4-dichlorophnoxyacetic acid) (8 combinations of treatments in total) in MS medium [29] to observe their effect on callus and somatic embryo formation in the study. The pH of the medium was adjusted without adding agar to 5.7±0.1 using 1 N KOH and 1 N HCl. The culture medium contained 6 g $l^{-1}$ (w/v) plant agar to solidify the medium. The somatic embryos were recovered on MS medium amended with 90 g $l^{-1}$ sucrose. All cultures were incubated under a 16 h light photoperiod using the light intensity of 2500±500 lux at 24±1ºC. The percentages of callus formation and embryo induction on the two explants were compared after 2 and 3 weeks of culture.

### Histological Procedure

Both leaf and stem explants inducing somatic embryos were initially fixed for 12-13 h using formalin acetic acid- ethyl alcohol fixative. They were dehydrated using ethyl alcohol and a series of butanol followed by embedding in paraffin and 10 μm microtomed sections. These were stained using safranin and fast green [30].

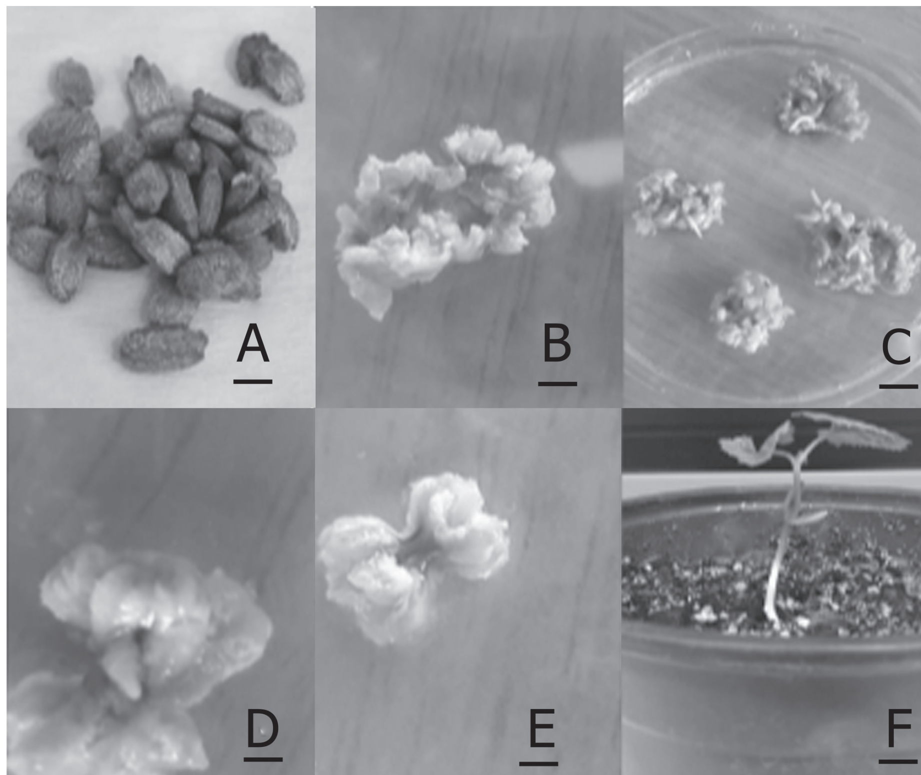


Fig. 1. A) Fresh seeds (B, C) Somatic embryogenesis on two-week old leaf explants in nutrient medium containing 0.5 mg l-1 $H_3BO_3$ and 2.0 mg l-1 2,4-D (D, E) Somatic embryogenesis on three-week-old stem explants in nutrient medium containing 1.5 mg l-1 $H_3BO_3$ and 2.0 mg l-1 2,4-D (F) Recovery of plant on MS medium and transfer to pots containing peat moss for acclimatization (bar Fig. 1A = 3.0 cm, Fig. B,C = 1.31 cm, 1D, E = 1.26 cm, 1F = 3.00 cm).

### Statistical Analysis

The experimental data was collected and analyzed according to split plot, randomized complete block design using three replications and repeated thrice. The measurements and counts were made on 15 explants in each replication. The data were subjected to variance analysis using the "SPSS 25.0 for Windows" statistical software. Duncan's test was applied to compare the treatment means. The LSD test at significance levels of "$p$<0.01 and $p$<0.05" were used to compare the means [31].

## Results and Discussion

### Histological Results

Histological sections showed the induction of somatic embryos was through formation of embryogenic clumps of cells induced on the explants.

### Effect of Different $H_3BO_3$ and 2,4-D Concentrations on *in vitro* Callus Production and Somatic Embryo Induction after Two Weeks of Culture

Somatic embryogenesis on the leaf explant started 2-3 d after culture with the initiation of callusing at the edges and formation of somatic embryos (after passing through different stages of globular, heart, torpedo, and well-formed plantlet stage after two weeks of culture). A significant interaction was noted among different concentrations of $H_3BO_3$, and 2,4-D for callus induction and somatic embryo regeneration on leaf and stem explants, which ranged 6.66-53.33% using 1.0 mg $l^{-1}$ 2,4-D and different concentrations of $H_3B0_3$ (Table 1). Somatic embryogenesis ranged 33.33-100.00% using 2.0 mg $l^{-1}$ 2,4-D on leaf explant using MS medium amended with different concentrations of (0.0-2.0 mg $l^{-1}$) $H_3BO_3$ (Table 1). The best somatic embryogenesis (100.00%) was noted on 0.5 mg $l^{-1}$ $H_3BO_3$ – 2.0 mg $l^{-1}$ 2,4-D (Fig. 1B, C).

Similarly, excluding non regenerative concentrations of $H_3BO_3$, a range of 13.33-33.33% somatic embrogenesis was noted using 1.0 mg $l^{-1}$ 2,4-D and 26.66-40.00% using 2.0 mg $l^{-1}$ 2,4-D in the presence of different concentrations of $H_3BO_3$ on stem explants. The best somatic embryogenesis (100.00%) was noted on 0.5 mg $l^{-1}$ $H_3BO_3$ – 2.0 mg $l^{-1}$ 2,4-D on leaf explant. No somatic embryogenesis was noted on stem explant using MS medium amended with 1.5 mg $l^{-1}$ $H_3BO$ – 1.0 mg $l^{-1}$ 2,4-D. No somatic embryogenesis was noted on 1.0 and 2.0 mg $l^{-1}$ $H_3B0_3$ combined with 2.0 mg $l^{-1}$ 2,4-D (Table 1).

Comparing the effects of two concentrations of 2,4-D, significant (*p<0.01*) differences were observed

Table 1. The effect of different concentrations of $H_3BO_3$ and 2,4-D on *in vitro* callus formation (%) and recovery of somatic embryos per explant from leaf and stem explants after two weeks of culture.

| $H_3BO_3$ (mg $l^{-1}$) | 2,4-D (mg $l^{-1}$) | | | | | Means |
|---|---|---|---|---|---|---|
| | Leaf | | Means | Stem | | |
| | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$) | | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$) | |
| Control | 6.66c**Bǂ | 86.66abA | 46.66c**** | 33.33a**Bǂ | 40.00aA | 36.66a**** |
| 0.5 | 33.33abB | 100.00aA | 66.665a | 20.00bB | 26.66bB | 23.33b |
| 1.0 | 46.66aB | 66.66bA | 56.66b | 13.33cA | 0.00cC | 6.67c |
| 1.5 | 20.00bB | 93.33aA | 56.66b | 0.00dB | 40.00aA | 20.00b |
| 2.0 | 53.33aB | 33.33cA | 43.33c | 26.66bA | 0.00cB | 13.33c |
| Means | 31.99B*** | 75.99A | | 18.66B | 21.33A | |
| Number of recovered somatic embryos explant-1 | | | | | | |
| $H_3BO_3$ (mg $l^{-1}$) | 2,4-D (mg $l^{-1}$) | | | | | |
| | Leaf | | Means | Stem | | Means |
| | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$) | | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$) | |
| Control | 0.76 | 9.89 | 5.34 | 0.81 | 0.97 | 0.88 |
| 0.5 | 3.71 | 11.33 | 7.62 | 0.43 | 0.64 | 0.54 |
| 1.0 | 5.33 | 7.61 | 6.97 | 0.32 | 0.00 | 0.16 |
| 1.5 | 2.28 | 10.65 | 6.47 | 0.00 | 0.97 | 0.49 |
| 2.0 | 6.09 | 3.81 | 4.95 | 0.64 | 0.00 | 0.32 |
| Means | 3.65 | 8.67 | | 0.45 | 0.52 | |

** The difference among the means shown with different lowercase letters in the same column are statistically different at $p<0.01$ level of significance using LSD test

ǂThe difference among the means shown with different uppercase letters in the same row are statistically different at $p<0.01$ level of significance using LSD test

*** The difference between the means shown with different lowercase letters in the same column are statistically different at $p<0.01$ level of significance using t test

**** The difference among the means shown with different uppercase letters in the same row are statistically different at $p<0.01$ level of significance using LSD test

between the treatments. 2.0 mg $l^{-1}$ was more inductive (with regeneration percentage of 75.99% on the leaf explant and 21.33% on stem explant after two weeks of culture (Table 1). Comparing the effects of different concentrations of $H_3BO_3$ both callus regeneration and somatic embryogenesis ranged 43.33-66.65% on leaf explants and 13.33-36.66% on stem explants.

## Effect of Different $H_3BO_3$ and 2,4-D Concentrations on *in vitro* Callus Production and Somatic Embryo Induction after Three Weeks of Culture

A significant interaction was noted among different concentrations of $H_3BO_3$ and 2, 4-D. Callus induction and somatic embryo regeneration ranged 6.66-40.00% using 1.0 mg $l^{-1}$ 2,4-D and 26.66-100.00% using 2.0 mg $l^{-1}$ 2,4-D in the presence of 0.5 mg $l^{-1}$ and 2.0 mg $l^{-1}$ $H_3BO_3$ on leaf explant in the same order (Table 2).

The best somatic embryogenesis (100.00%) was noted on 0.5 mg $l^{-1}$ $H_3BO_3$ (Fig. 1D, E). Comparing the effects of two concentrations of 2,4-D, significant (*p<0.01*) differences were observed between the treatments. A higher percentage of somatic embryos were induced on 2.0 mg $l^{-1}$ 2,4- D regardless of the explants. The leaf explant showed 69.99% and stem explants induced 23.33% somatic embryos. Comparing the effects of different concentration of $H_3BO_3$ both callus regeneration and somatic embryogenesis ranged 33.33-66.66% on leaf and 9.99-33.33% on stem explants. The results indicated statistically different results on the effects of $H_3BO_3$ and 2,4-D concentrations along with their interaction for somatic embryo induction on both explants. The highest percentage of somatic embryo induction (100.00%) on leaf explants was obtained on MS medium containing 0.5 mg $l^{-1}$ $H_3BO_3$ and 2.0 mg $l^{-1}$ 2,4-D with 93.33% somatic embryo induction on leaf explants on medium containing 1.5 mg $l^{-1}$ 2,4-D. The highest percentage of somatic embryo induction (60.00%) on stem explants

Table 2. Effect of different concentrations of $H_3BO_3$ and 2,4-D on *in vitro* callus formation (%) and recovery of somatic embryos per explant from leaf and stem explants after three weeks of culture.

| $H_3BO_3$ (mg $l^{-1}$) | 2,4-D (mg $l^{-1}$) | | | | | Mean |
|---|---|---|---|---|---|---|
| | Leaf | | Mean | Stem | | |
| | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$)** | | 1.0 (mg $l^{-1}$)* | 2.0 (mg $l^{-1}$)* | |
| Control | 6.66$^{b**B\phi}$ | 66.66$^{ab**A}$ | 36.66b$^{****}$ | 13.33$^{ab**AB\phi}$ | 40.00$^{abAB}$ | 26.66a$^{****}$ |
| 0.5 | 33.33$^{abB}$ | 100.00$^{aA}$ | 66.66a | 20.00$^{aAB}$ | 6.66$^{bAB}$ | 13.33b |
| 1.0 | 0.00$^{cB}$ | 60.00$^{bA}$ | 30.00b | 13.33$^{abAB}$ | 6.66$^{bAB}$ | 9.99b |
| 1.5 | 26.66$^{abB}$ | 93.33$^{aA}$ | 59.99a | 6.66$^{cAB}$ | 60.00$^{aA}$ | 33.33a |
| 2.0 | 40.00$^{aB}$ | 26.66$^{cA}$ | 33.33b | 6.66$^{cAB}$ | 3.33$^{cB}$ | 5.00b |
| | 21.33b$^{***}$ | 69.99a | | 11.99a$^{***}$ | 23.33b | |

| Number of recovered somatic embryos explant$^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|
| 2,4-D (mg l-1) | | | | | | |
| $H_3BO_3$ (mg $l^{-1}$) | Leaf | | Means | stem | | Means |
| | 1.0 (mg $l^{-1}$) | 2.0 (mg $l^{-1}$)** | | 1.0 (mg $l^{-1}$) | 2.01.0 (mg $l^{-1}$)** | |
| Control | 0.61 | 6.08 | 3.35 | 0.32 | 0.96 | 0.64 |
| 0.5 | 3.04 | 9.13 | 6.15 | 0.48 | 0.15 | 0.32 |
| 1.0 | 0.00 | 5.53 | 2.77 | 0.32 | 0.17 | 0.25 |
| 1.5 | 2.43 | 8.52 | 5.97 | 0.13 | 2.40 | 1.27 |
| 2.0 | 3.65 | 2.43 | 3.04 | 0.14 | 0.71 | 0.48 |
| Means | 1.95 | 6.39 | | 0.28 | 0.88 | |

** The difference among the means shown with different lowercase letters in the same column are statistically different at $p<0.01$ level of significance using LSD test

$^{\phi}$The difference among the means shown with different uppercase letters in the same row are statistically different at $p<0.01$ level of significance using LSD test

*** The difference between the means shown with different lowercase letters in the same column are statistically different at $p<0.01$ level of significance using t test

**** The difference among the means shown with different uppercase letters in the same row are statistically different at $p<0.01$ level of significance using LSD test

was obtained on MS medium containing 1.5 mg $l^{-1}$ $H_3BO_3$ and 2.0 mg $l^{-1}$ 2,4-D.

The callus induction on leaf explants ranged 0.00-40.00% and 26.66-100.00% using 1.0 and 2.0 mg $l^{-1}$ 2,4-D in the same order. The experimental results showed damaging effects of prolonged exposure (3rd week) on the health of leaf explants due to 2,4-D in the cultures on the number of somatic embryo formation ending up with a reduced number of somatic embryos regardless of using 1.0 and 2.0 mg $l^{-1}$ 2,4-D.

The stem explants induced improved callus and somatic embryogenesis of 3.33- 60.00% using 2.0 mg $l^{-1}$ 2,4-D, when 3 weeks results were compared with the data taken after two weeks of culture. The calli on both concentrations of 2,4-D induced hyperhydricity, on the control treatments, where $H_3BO_3$ was not used in the regeneration medium. The calli showed clumps of a multiple number of fused and hyperhydric somatic embryos that were difficult to separate in agreement with Navarro et al. [32] and Quiala et al. [33]. Qu [34], Vesty [35] recommend the use of a medium containing $H_3BO_3$ and 2,4-D at different concentrations to induce callus induction and somatic embryo formation. Studies by other researchers also recommend the "short interval treatments" of $H_3BO_3$ and other plant growth regulators to culture bitter gourd and other plants [36-41]. Plantlets were obtained from somatic embryos *in vitro* conditions and were acclimated to *in vivo* conditions in pots in climate chamber (Fig. 1F).

## Conclusions

It was concluded that short-term (two or three weeks) cultures are more advantageous for *in vitro* callus and somatic embryo formation of *M. charantia*. Media containing the combination of 2,4-D and $H_3BO_3$, which produced significantly different results on leaf and stem explants. The study reports tissue culture of bitter gourd from a local line/ecotype grown in West Asia or Turkey for the first time. This study could be extended further for genetic transformation, genetic

editing, rapid breeding and secondary metabolite production. Furthermore, the regeneration was explant and time-dependent. The process of regeneration varied depending on the capability of explants to induce new meristems. The study meets the objectives of the study.

## Conflict of Interest

The authors declare no conflict of interest.

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