Efﬁcient shoot induction from apical meristem culture in olive (*Olea europaea* L.)

ABSTRAC

Olive propagation by tissue culture method is an efficient method for rapid asexual propagation, production of disease-free plants, and access to propagating materials throughout the year for olive plants. In this study, meristematic tissue explants of the Koroneiki cultivar from olive trees were cultured in different basal media included MS (Murashige and Skoog), OM (Olive Medium) and DKW (Driver and Kuniyki), without plant growth regulators. Then, the explants were cultured in basal media supplemented with auxins [NAA (Naphthalene acetic acid, IAA (Indole acetic acid) and IBA (Indole butyric acid)] and cytokinins [BA (Benzyl adenine), KIN (Kinetin), 2ip (Isopentenyl adenine), TDZ (Thidiazuron), and ZEA (Zeatin)] at concentrations of 0.1 to 4 mg/l The superiority of DKW basal media and ZEA growth regulator for inducing branching and longitudinal growth of branches for the Koroneiki cultivar was proven. With increasing concentrations of growth regulators, especially BA, was the dominant response than other responses such as meristem growth and shoot induction.

Key Words: Apical meristem, Driver and Kuniyki medium, Olive, Zeatin

1. INTRODUCTION

The olive (*Olea europaea* L.) tree is one of the oldest and the most symbolic cultivated plants in the Mediterranean region. The annual yield of olive is estimated at 10 million tons, most of which is used for oil production and less than 10% consumed as table olive. As regards the world trend in the last 30 years, production and consumption of olive oil have increased together. It is unlikely that this trend will change in the near future, considering the recent introduction or increase of olive cultivation and olive-oil consumption in countries such as Japan, Australia, China and South Africa. The traditional area of olive cultivation is the Mediterranean basin, which accounts for 95% of the olive orchards of the world, and where, mainly in Spain, Italy and Greece, almost 99% of the world’s olive oil and more than 80% of its table olives are produced IOOC, (2001); Jain and Ishii, (2003).

One of the serious threats to olive trees is olive diseases, especially viral diseases. The viruses cause severe diseases resulting in great yield losses and reduced olive quality. Elimination plant viral agents through meristem culture, which is considered one of the methods of plant cell, tissue, and organ culture, is one of the best methods for producing virus-free plants and plant materials Alam et al. (2004). The plants propagated by this method often have faster growth, higher resistance to plant diseases, higher quality, and greater reproductive capacity Yahyaoui et al., (2021).

Given that olive propagation is often asexual (cuttings), pathogens are easily transmitted through propagation materials such as rootstocks, scions, and cuttings, that is caused to contamination of orchards. In fact, Olive trees are mainly propagated using semi-hardwood cuttings. This procedure has contributed over the years not only to the dissemination of the best olive materials, but also to the spread of pathogens, predominantly viruses Xylogianni et al., (2021). Using healthy and genetically authentic scions, is one of the most important and main steps to control viral contamination. As mentioned above, one of the effective methods for defense against viral contamination of plants is the meristem culture method.

There are also other methods, such as thermotherapy and chemotherapy, to combat plant viruses. These methods and the meristem culture method are often used individually or together against viral diseases of plants.

The goal of the present study is to optimize the culture method of the meristem zone of olive young branches in order to achieve methods for producing olive virus-free plants using the meristem culture method.

1. MATERIAL AND METHOD

2.1 Plant material and Sterilization

The plants tested in this study, which included 4-year-old scions of olive, were purchased from Fadak Garden, located in Qom province, 15 km from the old Qom-Kashan Road. The scions were placed in the greenhouse of Iranian Research Institute of Plant protection. For preparation of explants, the apical parts of the (young) branches were cut and the first one centimeter of their apex was separated and sterilized as follows. Due to the thinness and high sensitivity of tissues, diluted commercial sodium hypochlorite was used to sterilize them as follow:

1- 60 minutes, 1% sodium hypochlorite

2- 30 minutes, 2% sodium hypochlorite

3- 15 minutes, 3% sodium hypochlorite

Then, the explants were washed 4 to 5 times with sterile distilled water to completely eliminate the effect of the sterilizing agent (sodium hypochlorite). The explants were cultured on free hormone medium included MS Murashige and Skoog (1962), DKW Driver and Kuniyki (1984) and OM Rugini (1984). Then a 0.8 to 1 mm portion from completely sterilized meristematic zone of young branches was separated with a sterile scalpel and cultured on media supplemented with Plant Growth Regulators as: [1 to 4 mg/l BA, KIN, 2ip, TDZ and ZEA from cytokinin(s) and 0.1 to 0.4 mg/ IBA from auxin(s)]. All media contained 30 g/L sucrose and 7.5 g/L agar. Media pH was adjusted to 5.8 before adding agar and before autoclaving (120°C and 1.5 atmosphere) using HCl and NaOH 1N. Cultures incubated at 25 ± °C with a 16 h photoperiod provided by cool white fluorescent lamps (38mol/M2S).

* 1. Statistical analysis

Experiments arranged in a randomized complete block design, with 28 explants (4 petri dish each with 7 explants) per treatment with four replications. Observation was based on, the mean number of explants (callus or regenerated shoot) reactant. statistically analysis was done using Duncan’s multiple range tests.

1. RESULT

3.1 Establishment of explants

The results of sterilizing olive explants, which include delicate and sensitive tissues of the plants branches (including meristem and meristematic tissues), showed that the use of commercial sodium hypochlorite (2%) for 30 minutes was more suitable than other treatments, because the percentage of sterilization and survival of explants was higher. The effect of different concentrations of commercial sodium hypochlorite and its different exposure times on olive explants is summarized in Table 1.

|  |  |  |  |
| --- | --- | --- | --- |
| Sterilization and survival  (%) | Exposure time  (minutes) | Hypochlorite sodium (%) | Sterilizing treatment |
| 85 | 60 | 1 | 1 |
| 98 | 30 | 2 | 2 |
| 95 | 15 | 3 | 3 |

Table 1. Sterilization of olive explants using commercial sodium hypochlorite

As mentioned in the Materials and Methods section, these sterilized tissues were first cultured in hormone-free basal culture media (MS, OM and DKW) In order to control contamination as much as possible, after ensuring that the tissues were sterile, the 0.8 to 1 mm distal sections were separated under sterile conditions in a laboratory hood and transferred to the above media supplemented with plant growth regulators or plant hormones (Fig 1).

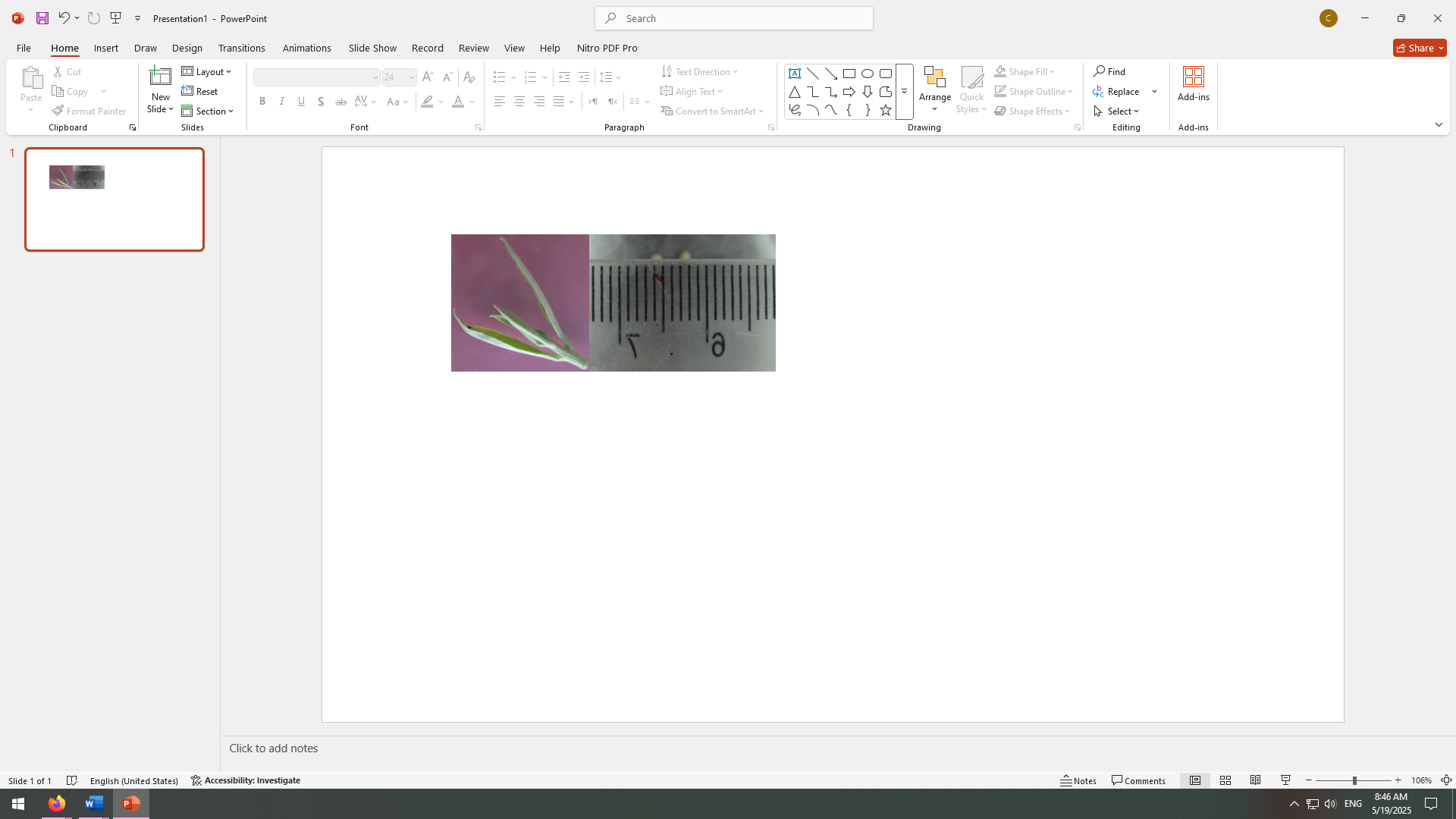


Fig 1. Steps for preparing and culture olive meristem tissues in hormone-free culture media

3.2 The effect of basal culture media and different plant growth regulator treatments on (continued) meristem growth callus and shoot induction

After successfully establishing the explants in the culture media, the effect of basal media as well as the kind and concentration of plant Growth Regulator was very clear. This effect was most evident on callus formation Fig. 2, meristem growth, and shoot induction.

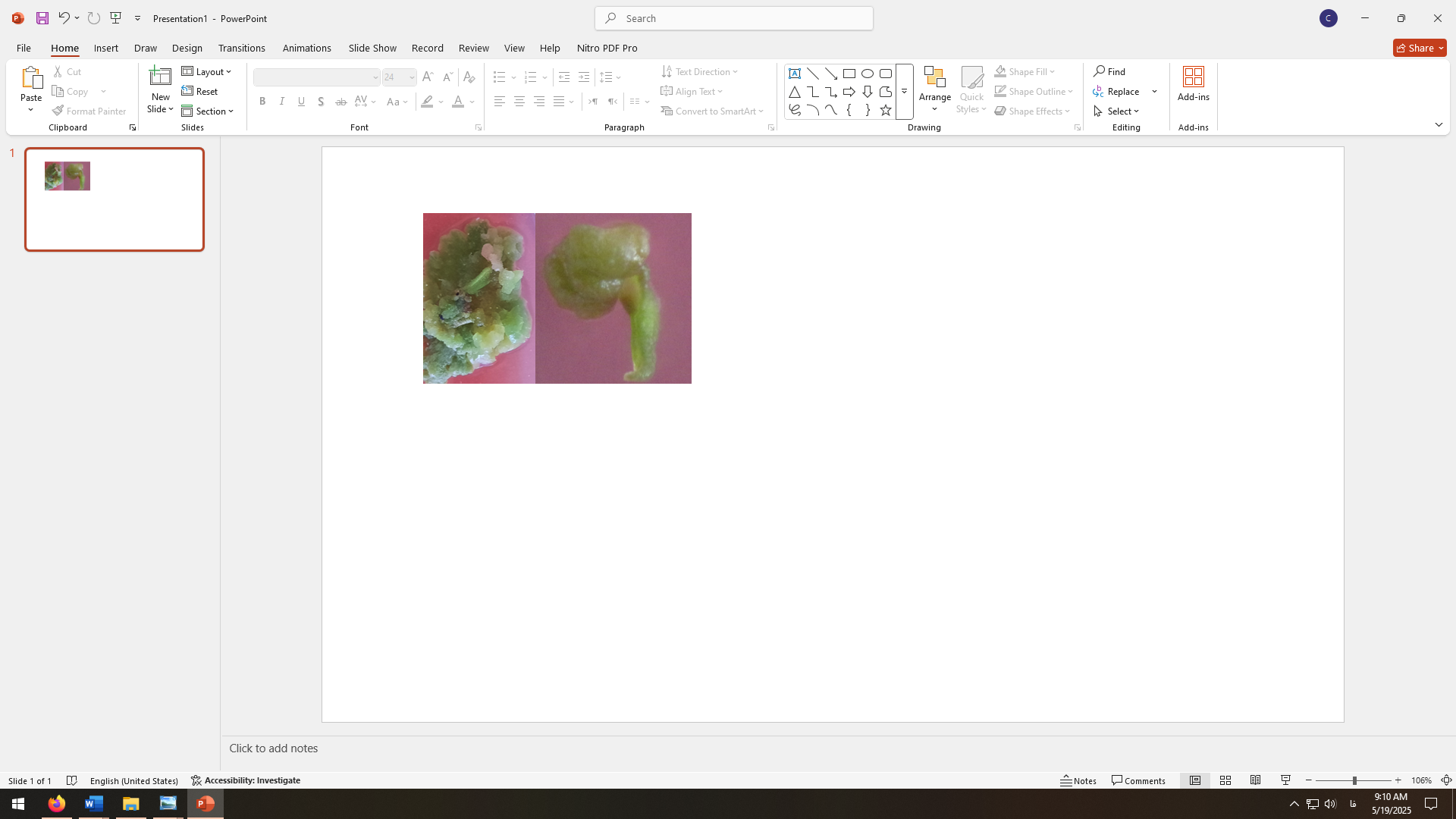


Fig 2. Callusing in DKW medium with 2 mg/l BA and 0.2 mg/l IBA

The results showed that, the effect of cytokinin(s) and their concentrations started from a minimum amount, then increasing the concentration, increased the response somewhat and concentrations greater than the maximum (used in this study) decreased the response. Addition of 3 mg/L ZEA with or without 0.3 mg/l IBA had the effect on meristem growth and plantlet formation (aerial organ) in terms of the number of explants showing response (9a (Table. 2).

Other cytokinins had less effect in terms of both the initiation of the reaction and shoot regeneration from callus differentiation. Also, in all the treatments studied, ZEA caused the longitudinal growth of shoots to a greater extent than other cytokinins, with the exception of media containing 2ip that the longitudinal growth of the resulting shoots was greater than that of other treatments (data were not shown). Also, KIN and 2ip had obvious and positive effects on main factors examined in this study (callus induction and shoot regeneration from differentiation of callus tissues) respectively.

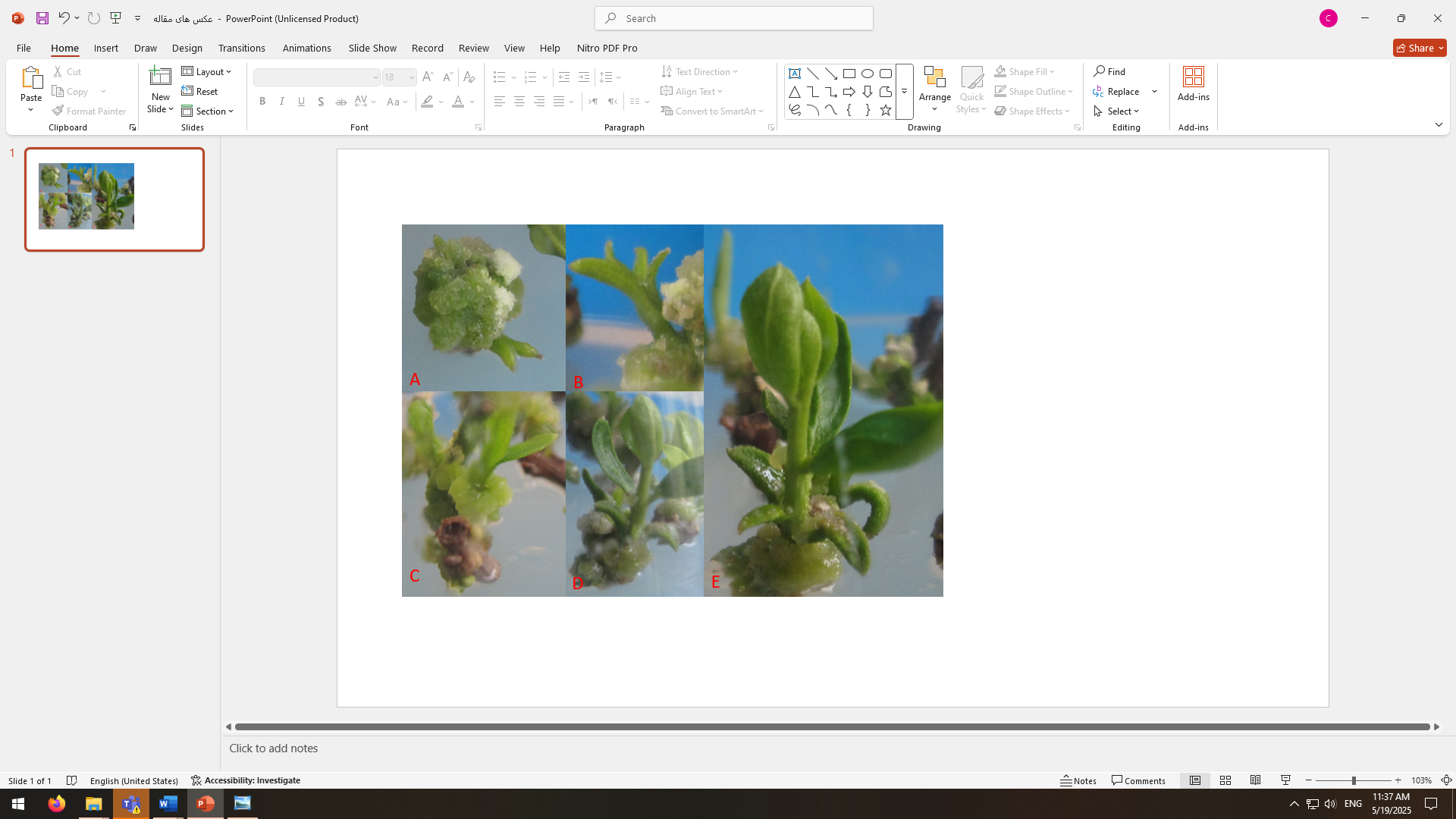
Different concentrations of TDZ used had no obvious effect on meristem growth, callusing and shoot formation. It is important to note that the positive effect of growth regulators ZEA and KIN on DKW medium and 2ip on OM medium were studied. In the MS basal medium, shoot regeneration were also observed with plant meristem culture. In MS basal medium, the most effective growth regulator for the desired reactions was BA. Another difference between the MS basal medium enriched with BA was the greater growth of calluses after the explants were established in the culture media. Because in the present study the best responses were observed in DKW basal medium and most of the explants with callusing and shoor regeneration belonged to it, only the effect of this basal medium enriched with cytokinins and auxins is summarized in table 2 and Fig.3 (A – E).

Figure 3 (A- E) A- Establishment of olive meristem in DKW medium containing 3 mg/L ZEA. B and C - in DKW medium containing 4 mg/L ZEA D - in DKW medium containing 4 mg/L KIN and E- In DKW medium containing 4 mg/l ZEA

Table 2. The mean number of explants (with new shoots or calli) on DKW basal media experimented

|  |  |  |  |
| --- | --- | --- | --- |
| Percent response | Concentrations  (mg/l) | Plant Growth Regulators | Treatment number |
| 1.9def | 1 + 0.1 | BA + IBA | 1 |
| 1.8def | 2 + 0.2 | BA + IBA | 2 |
| 1.9def | 3 + 0.3 | BA + IBA | 3 |
| 2.5cdef | 4 + 0.4 | BA + IBA | 4 |
| 4.75bcd | 1 + 0.1 | ZEA + IBA | 5 |
| 7.5ab | 2 + 0.2 | ZEA + IBA | 6 |
| 9a | 3 + 0.3 | ZEA + IBA | 7 |
| 3.9cde | 4 + 0.4 | ZEA + IBA | 8 |
| 1.7def | 1 + 0.1 | 2ip + IBA | 9 |
| 1.7cde | 2 + 0.2 | 2ip + IBA | 10 |
| 3.5cde | 3 + 0.3 | 2ip + IBA | 11 |
| 4.4bcd | 4 + 0.4 | 2ip + IBA | 12 |
| 0f | 1 + 0.1 | TDZ + IBA | 13 |
| 0f | 2 + 0.2 | TDZ + IBA | 14 |
| 1.12ef | 3 + 0.3 | TDZ + IBA | 15 |
| 1.02ef | 4 + 0.4 | TDZ + IBA | 16 |
| 4.6bcd | 1 + 0.1 | KIN + IBA | 17 |
| 7.37ab | 2 + 0.2 | KIN + IBA | 18 |
| 5.6bc | 3 + 0.3 | KIN + IBA | 19 |
| 1.6def | 4 + 0.4 | KIN + IBA | 20 |
|  |  |  |  |

Means values within a column with different letters are significantly different at P=0.015 according to Duncan’s Multiple Range Test

1. DISCUSSION

The reason for using the meristem culture method in this project and other research conducted with this goal (production of healthy plant materials) is utilization the characteristic of the shoot apical meristem. This characteristic is the totipotency of shoot apical meristem cells. It is important that, all of the shoot apical meristem cells have not similar totipotency. The highest level of totipotency is observed in leaf generative centers. So, depending on the size of the isolated tissue and incision place, different reactions occur, from callus formation to callus differentiation into stem and root, complete plantlet, and meristem repair. With cut of 0.1 to 0.5 mm from the meristem apex, a little callus is formed at first, and then stem and root are formed from the callus differentiation

The totipotent cells of this organ due to very little differentiation and incomplete differentiation of vascular tissues and also the lack of some cell surface receptors which leads to plant recognition microbial agents, are used to produce plants free of any pathogen Wang and Charles (1991), Gulan (2007) and Ball (1960).

The results showed that by separating 0.8 to 1 mm sections of meristem apex and to culture them on media, first callus and then aerial organs were produced.

Siripatr et al. (2011), observed a shoot-forming response by culturing the meristem of passion flower (*Passiflora edulis*) on MS medium containing 1 to 3 mg/L BA. The above researchers used MS medium containing 0 to 1.2 mg/L IBA for rooting of regenerated shoots. The results of these researchers differ from the present study in terms of the lack of callus formation before the development of aerial organs.

Another study on strawberry (*Fragaria chiloensis* (L.) Duch.), showed that on MS basal medium enriched with BA shoot induction is predominant reaction. In this study, the presence of BA in the medium increased shoot formation also, its usage, had low levels (near zero) of explant losses due to oxidation. Quiroz et al., (2017).

Yao et al. in (2022) stated that for induction of new shoots (by passing through the callus phase) of *Salvia miltiorrhiza* (a type of sage) on MS medium, application of 0.5 mg/L BA, 0.1 mg/L NAA, and 0.1 mg/L GA3 is essential. The researchers used 0.5 MS medium and 1 mg/L NAA to root the new shoots. They also noted that calli generated from the shoot apical meristem are superior to other plant tissues and aerial organs in terms of their induction and production of new organs. This research work is agreement with the present research in terms of callus induction immediately after meristem culture.

The positive effect of ZEA and BA on growth and formation of aerial organs from meristems of Koroneiki cultivar olive, compared to other cytokinins used in this study, was another important result of the experiments. The growth regulator BA plays a very important role in all cell, tissue and plant organ culture research, with emphasis on effect of it on shoot induction (newly emerging branches).

There is also a lot of evidence about the positive role of ZEA, which confirms the results of this project, for example, Benelli and De Carlo in 2018 stated that 10 µ ZEA not only case to induction of olive apical buds but also causes longitudinal growth new shoots. (Benelli and De Carlo, 2018).

Another report has shown that 4/18 mµ ZEA has a negative effect on olive growth factors (Haddad et al., 2018). Obviously, this conclusion does not confirm the results of this project.

Ali et al. (2009) showed that 4 mg/L ZEA caused a significant increase in shoot formation in olive plants compared to concentrations of 1, 2, and 3 mg/L.

In 2021, researchers pointed to the positive growth-regulating effect of ZEA in different MS and OM basal media for Arbaquin and Muscat olive cultivars Mirzaei et al., (2021). The mentioned subject shows that even on other basal media (compared to the DKW basal media used in this study) such as MS and OM which have been used for tissue culture of other olive cultivar, ZEA plays an important role.

CONCLUSION

According to the gardeners and olive scions’ growers, tissue culture is a superior and preferred method for olive propagation due to the difficulties for the traditional methods especially difficulties related to different methods for rooting of different cuts olive cultivars. Meristem culture is both a way to overcome traditional propagation methods of this plant and an introduction virus-free olive plants production.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author hereby declares that NO generative AI technologies such as Large Language Models

(ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

References

Alam, MF., Banu, M.L.A., & Swaraz, A.M et al. (2004). Production of virus free seeds using meristem culture in tomato plant under tropical conditions. *Journal of Plant Biotechnology,* 6 (4), 221 – 227.

<http://www.kspbt.or.kr>

Ali, A., Ahmad, T., Abbasi, N.A., & Hafiz, I.A. (2009). Effect of different media and growth  
regulators on in vitro shoot proliferation of olive cultivar ’moraiolo’. Pakistan Journal of Botany. <http://www.pakbs.org//contents.htm>

Ball, E. (1960). Cell divisions. In living shoot apices. Phytomorphology. 10, 377 – 396

Benelli, C., & De Carlo, A. (2018). In vitro multiplication and growth improvement of Olea europaea L. cv Canino with temporary immersion system (PlantformTM). 3 Biotech.

https:// doi. org/ 10.1007/ s13205- 018- 1346-4.

Galun, E. (2007). [Plant Patterning: Structural and Molecular Genetic Aspects](http://www.worldcat.org/title/plant-patterning-structural-and-molecular-genetic-aspects/oclc/137324936&referer=brief_results). World Scientific Publishing Company, 333. [ISBN](https://en.wikipedia.org/wiki/ISBN_(identifier)) [9789812704085](https://en.wikipedia.org/wiki/Special:BookSources/9789812704085)

DOI:[10.1142/6326](http://dx.doi.org/10.1142/6326)

Haddad, B., Carra, A., Saadi, A., Haddad, N., Mercati, F., Gristina, A. et al. (2018). In vitro propagation of the relict laperinne’s olive (*Olea europaea* L. subsp. Laperrinei). Plant Biosystems. (2018). https:// doi.org/10.1080/11263504.017.13060 02

Driver, J.A., Kuniyki, A.H. (1984). In vitro propagation of paradox walnut Rootstock. *Horticulture Science*, 19(4), 507 – 509.

DOI: <https://doi.org/10.21273/HORTSCI.19.4.507>

IOOC. (2001). International Olive Oil Council. <http://www.internationaloliveoil.org/>, Economics, Production.

Jain, S.m., & Ishii, K. (2003). Micropropagation of Woody Trees and Fruits. *KLUWER Ac. Pub*, 621 – 646.

<http://www.researchgate.net/publication/251410896>

Mirzaei, L., Yadollahi, A., Jafarkhani Kermani, M., Naderpour, M., Zeinanloo, A.A., Farsi, M., et al. (2021Evaluation of genetic stability in olive callus – induced and meristem – induced shoots using flow cytometry and amplified fragment length polymorphism techniques. Plant Methods, 17(31), 1 – 15

<https://doi.org/10.1186/s13007-021-00724-7>

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco cultures. *Physiol Plant*, 15, 473– 497. <http://doi.org/1111/j.1399-3054.1962.tb08052.x>

Quiroz, K.A., Berrios, M., Carrasco, B., Retamales, J.B., & Garcia-Gonzales, C.R. (2017). Meristem culture and subsequent micropropagation of Chilean strawberry *(Fragaria chiloensis* (L.) Duch.). Biological Research, 50(20), 1 – 11

DOI 10.1186/s40659-017-0125-8

Siripatr, P., Sarut, T., Pissawan, C., Narongchai, P., & Pipattanawong, N. (2011) Efficient shoot regeneration from direct apical meristem tissue to produce virus – free purple passion fruit plants. Crop protection, 30, 1425 – 1429

doi: 10.1016/j.cropro.2011.07.008

Rugini, E. (1984). In vitro propagation of some olive (*Olea europaea sativa* L) cultivars with different root – ability and medium development using analytical data from developing shoots and embryos. Sci Hortic, 24, 123–134.

<https://doi.org/10.21273/HORTSCI.19.4.507>

Wang, P. J. & Charls, A (1991). Micropropagation through Meristem Culture. Biotechnology in Agriculture and Forestry, Vol. 17 High-Tech and Micropropagation I (ed. by Y.P.S. Bajaj)  
 Springer-Verlag Berlin Heidelberg

https://doi.org/10.1007/978-3-642-76415-8\_3

Xylogianni, E., Margaria, P., Knierim, D., Sareli, K., Stephan Winter, S., & K. Chatzivassiliou, E.k. (2021). Virus Surveys in Olive Orchards in Greece Identify Olive Virus T, a Novel Member of the Genus Tepoviru. *Pathogens*, 10, 574.

DOI: [10.3390/pathogens10050574](https://doi.org/10.3390/pathogens10050574)

Yahyaoui, E., Marinoni, D.T., Botta, R., Rufa, P., & Germana, M.A. (2021). Is it possible to produce certiied hazelnut plant material in sicily? Identification and recovery of nebrodi genetic resources, in vitro establishment, and innovative sanitation technique from apple mosaic virus. *Front Plant Sci,* 12, 778142

DOI: [10.3389/fpls.2021.778142](https://doi.org/10.3389/fpls.2021.778142)

Yao, S.C., Jiang, Y.Y. N., Wang, S., Feng, L, Rui, j., W. Yang, et al. (2022). Development of a highly efficient virus – free regeneration system of *Salvia miltiorrhiza* from Sichuan using apical meristem as explants. Plant Methods. 18(50), 1 – 12

https://doi.org/10.1186/s13007-022-00872-4