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Original paper

Preliminary research on getting callus in Ginkgo biloba L.

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Abstract

The purpose of such preliminary research has been represented by the establishment of the influence of treatments by growth regulators on Ginkgo biloba L. explants (taken from plants in their second year of vegetation), so as to get callus.

Therefore, one has tested the influence of three such growth regulator treatments (6-benzylaminopurine, 6-benzylaminopurine and α-naphthaleneacetic acid, or simply α-naphthaleneacetic acid), on nine types of cauline explants for the following purposes: pre-initiation of callogenesis, inducing and generating the callus, callus development and maintaining such callus development.

The best influence in order to get callus in Ginkgo biloba L., has been achieved in the case of the experimental variant consisting of the mixture between the treatment by α-naphthaleneacetic acid and the explants of the type taken from position 4 from rosette (MBAV5).

Keywords : Ginkgo biloba L., 6-benzylaminopurine, α-naphthaleneacetic acid, callus

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Introduction

In terms of Ginkgo biloba L. the current taxonomic classification is as follows: Plantae Kingdom, Gymnosperms Phylum, Ginkgoaceae Family, Ginkgo L type, Ginkgo biloba L. Species (http://www.theplantlist.org/tpl1.1/record/kew-334053 [1]).

The medical and economic relevance of Ginkgo biloba L. species is well renowned and is mainly based upon the use of leaves in various products (e. g., pills, tea, powders, juice, ointments, lotions and shampoo).

Murashige and Skoog (1962) basal nutritive medium - T. Murashige& F. Skoog [2] - supplemented by various growth regulators has proven to have a significant influence on the quality and proliferation of callus in plants.

In terms of Ginkgo biloba L., as early as of 1991, one has grown Ginkgobiloba L. cells on MS basal nutritive medium (1962), duly supplemented by 1.0 mg/l NAA and 0.1 mg/l KIN in 500 ml vases, or in bio-reactors with immobilization and the volume of 2 l or 6 l (D.J. Carrier & al. [3]). Yet, the quantity of ginkgolid B has been low in order to positively confirm the presence of this particular product. Also in terms of Ginkgo biloba L. one has tested the getting of callus from various types of initial inoculi (e. g., seeds, embryos, cotyledon tissue and embryos with removed cotyledons), grown on MS basal nutritive medium (1962), duly supplemented by 2.5 g/l NAA or by 2.5 g/l 6-BAP. However, one has got callus only from the inoculi of the embryo or cotyledon type. And the largest amount of callus has been got from the inoculum of the cotyledon type under the influence exercised by NAA 2.5g/l (https://link.springer.com/article/10.1023/B:BIOP.0000033460.75432.d1 [4]).

In 2009, Hao and the collaborators have brought proof to the fact that nitric oxide from an exogenous source triggers a growth in the latter's bio-synthesis activity if added to cultures of callus from Ginkgo biloba L., which growth has caused a better signaling in terms of: phenylalanine ammonium lyase activity coordination, flavonoids biosynthesis and stress tolerance (G. Hao & al. [5]).

Treating the cultures of Ginkgo biloba L. cells in suspension by endophytic fungi has triggered some beneficial effects, such as: the accumulation of flavonoids and the increase of abscisic acid production (G. Hao & al. [6]).

In 2014, from the callus induced from the embryos obtained from Ginkgo biloba L. seeds, that were in the cotyledon stage and which were grown on MS (1962) environment, duly supplemented by 2.0 mg/l NAA and 1 mg/l 6-BAP, one has extracted flavonoids and terpene lactones (S. CHENG & al. [7]).

Starting from foliar explants obtained by the due fragmentation of young leaves taken in august from mature speciments of Ginkgo biloba L., one has established some cultures of immobilized cells in Ginkgo biloba L. The latter have been achieved by growing 2 g of friable callus on 40 ml of MS (1962) basal nutritive medium, duly supplemented by 2.0 mg/l NAA and 0.1 mg/l KIN, at a temperature of 25ºC, under agitation at 100 rpm and incubation in the dark. In the presence of the jute fibres and under the synergetic effect of salicylic acid and of methyl jasmonate, such cultures of immobilized cells have generated conditions which seem to be optimum for the production of in vitro ginkgolides and bilobalides (A. SUKITO & S. TACHIBANA [8]).

Considering a large amount of information from the specialized literature (e. g.: getting callus in Ginkgo biloba L. especially from embryo structures; each cell of a zygotic type is unique; the biosynthesis of certain substances is dependant upon the age of the relevant source-plant, the stage of vegetation, the phytosanitary state; the tendency to use cell cultures for the biosynthetisation of certain substances of a pharmaceutical interest in view of replacing the acknowledged source, etc.), we found it necessary for someone to conduct some researches into the subject so as to enable both the valorization of the unicity of Ginkgo biloba L. plants obtained from zygotic type seeds and the accomplishment of biochemical or /and genetic tests which shall highlight the profile of such unicity.

As a consequence, considering the aforementioned, the purpose of such preliminary research has been represented by the establishment of the influence of the treatment by growth regulators on the Ginkgo biloba L. explants (taken from plants obtained by the germination of the previous year seeds and in a state of vegetation), in order to get callus. Basically, one has tested the influence of three treatments by growth

regulators (6-benzylaminopurine, 6-benzylaminopurine and α-naphthaleneacetic acid, or simply α-naphthaleneacetic acid), on nine types of cauline explants for the following purposes: pre-initiation of callagenesis, inducing and generating the callus, callus development and maintaining such callus development.

Materials and Methods

The biological material and the work methodology have complied with the criteria that are well acknowledged in the vegetal biotechnologies field, namely: the biological material shall be under good phytosanitary condition and the work methodology shall be characteristic to the in vitro work conditions.

The primary source of explants has consisted of Ginkgo biloba L. plants obtained out of the germination of seeds from the previous year and which were in the vegetation period (spring).

For the purpose of conducting the pre-aseptizationat the surface, the Ginkgo biloba L. explants have been washed by continuous coldwater jet for 30 minutes and then immersed into an 80% sodium hypochlorite solution (B. E. MOGHADDAM & al. [9]), for 15 seconds. Then, for the same purpose of surface aseptization, the explants have been immersed into 1.5% sodium hypochlorite solution (B. E. MOGHADDAM & al. [9]), for 10 minutes. After the aseptization, the explants required three successive washing operations (of 10 minutes each), in aseptized distilled water (E. M. BADEA & D. SĂNDULESCU [10]; D. CACHIȚĂ-COSMA& al. [11]).

The basal nutritive medium Murashige Skoog (1962) - T. Murashige & F. Skoog [2] -, has been duly supplemented by two types of growth regulators: 6-benzylaminopurine and α-naphthaleneacetic acid (D.-J. Carrier & al., [3]; https://link.springer.com/article/10.1023/B:BIOP.0000033460.75432.d1 [4]; S. CHENG & al. [7]; A. SUKITO & S. TACHIBANA [8]). Basically, one has prepared 3 types of nutritive mediums that were based on the basal nutritive medium Murashige and Skoog (1962), duly supplemented by:

- 6-benzylaminopurine (2 mg/l) = *MB treatment*,
- 6-benzylaminopurine (1 mg/l) and α-naphthaleneacetic acid (1 mg/l) = *MBA treatment* and
- α-naphthaleneacetic acid (2 mg/l) = *MA treatment*.

Prior to autoclavation, the pH has been adjusted at the 5.3 value. The nutritive mediums aseptization has been carried out by autoclavation, at a temperature of 121°C, for 15 minutes.

The *culturing conditions* have been achieved by incubation in a device of the Sanyo type (Versatile environmental test chamber), at a temperature of 21°C ± 2°C, for the photoperiod of 16 hours and the air relative humidity of 70%.

The *experimental plan* has included the mixture of the three treatments, as above described (MB treatment, MBA treatment and MA treatment), with nine experimental variants for the explants type under focus. The nine experimental variants have consisted of cauline explants of the following type:

- apical bud + one part of the stem = V_1;
- leaf prelevated from the position 1 in the rosette = V2;
- leaf prelevated from the position 2 in the rosette = V3;
- leaf prelevated from the position 3 in the rosette = V4;
- leaf prelevated from the position 4 in the rosette = V5;
- leaf with detached sides prelevated from the position 5 in the rosette = V6;
- leaf damaged by longitudinal sectioning prelevated from the position 6 in the rosette = V7;
- apical leaf section prelevated from the position 7 in the rosette = V8 and
- basal leaf section prelevated from the position 7 in the rosette = V_9.

Practically, the experimental plan consisted of twenty-seven experimental variants.

The methods of analysis of the biological material consisted of morphometric determinations (the diameter of hyper-hydrated explant and the diameter of callus).

Statistical procedures. Each experimental variant consisted of 3 repetitions and the experiments were repeated twice. Measurements were made for each individual inoculum. The data were statistically analysed and the standard deviation of mean was calculated.

Results and Discussions

A. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the pre-initiation of callogenesis

A.1. The preliminary testing of the influence exercised by the treatment by 6-benzylaminopurine on *Ginkgo biloba L.* explants for the pre-initiation of callogenesis has triggered the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 1).

Based upon the experimental results shown in Figure 1 can see that the experimental variant MBV2, which has included the treatment by 6-BAP and explants of the leaf taken from the position 1 in the rosette, has triggered the highest average value of the diameter of hyper-hydrated explants. Whereas, the experimental variant MBV9, which consisted of the treatment by 6-BAP and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of hyper-hydrated explants.

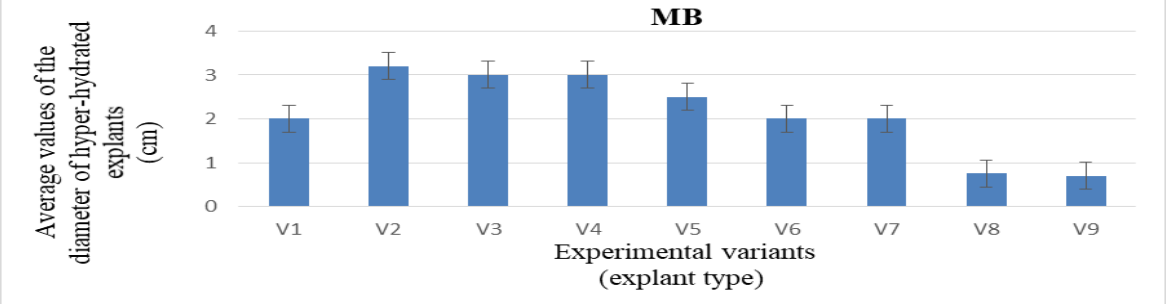

Figure1. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by 6-benzylaminopurine, subject to experimental variants

A.2. The preliminary testing of the influence exercised by the treatment by 6-benzylaminopurine and α-naphthalene acetic acid on Ginkgo biloba L. explants, for the pre-initiation of callogenesis has triggered both the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 2), which phenomenon has also been noticed in the case of the treatment by 6-BAP, as well as the intensification of the green color of explants.

Given the experimental results displayed in Figure 2 it follows that the experimental variant MBAV2, which consisted of the treatment by 6-BAP and NAA, and of explants of the leaf type taken from the position 1 in the rosette, has enabled the occurrence of the highest average value of the diameter of hyper-hydrated explants.

And the variant MBAV8, which included both the treatment by 6-BAP and NAA, and explants of the apical leaf section type taken from the position 7 in the rosette, has enabled the occurrence of the lowest average value of the diameter of hyper-hydrated explants.

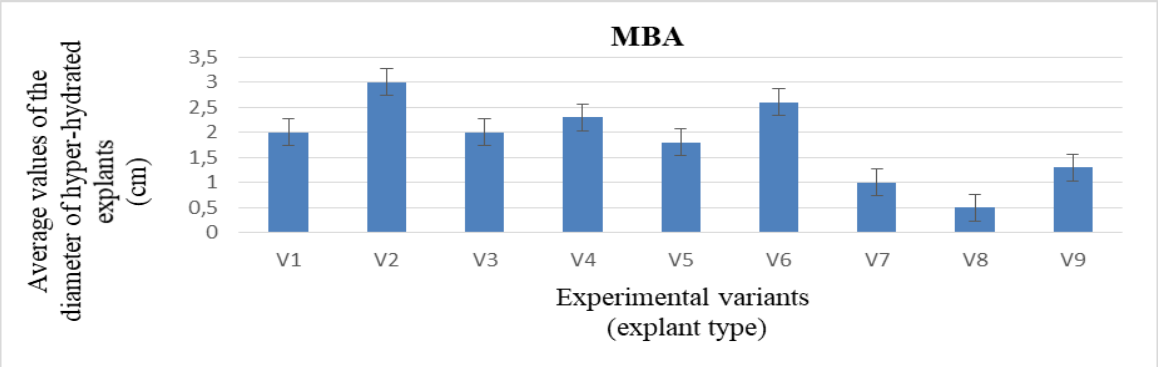

Figure 2. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

A.3. The preliminary testing of the influence exercised by the treatment by α-naphthalene acetic acid on Ginkgo biloba L. explants, for the pre-initiation of callogenesis has triggered both the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 3), which phenomenon has also been noticed in the case of the other two treatments.
However, one has also noticed the intensification of the green color of explants, although such color has only be seen in the case of the treatment by 6-BAP and NAA.

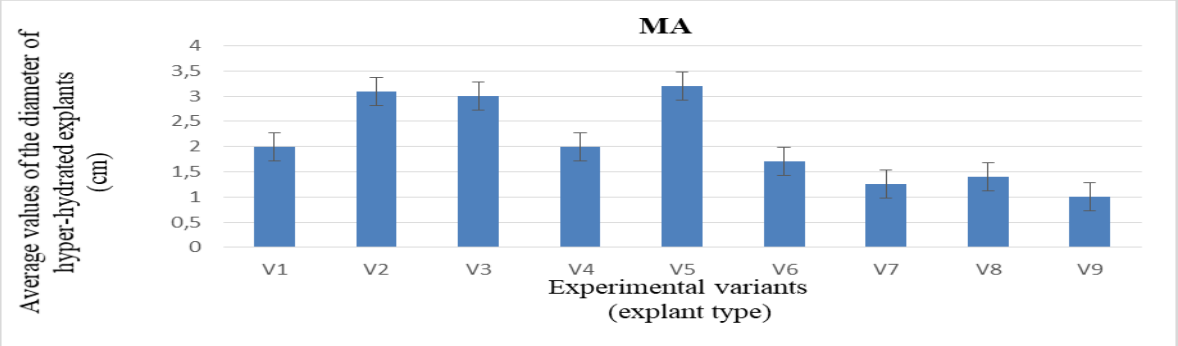

Figure 3. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by α-naphthalene acetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 3 can see that the experimental variant MAV5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of hyper-hydrated explants. Whereas, the experimental variant MAV9, which consisted of the treatment by NAA and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of hyper-hydrated explants.

Reviewing the experimental results displayed in Figure 1, Figure 2 and Figure 3, one may notice that the preliminary testing of the influence of the treatment based on growth regulators on *Ginkgo biloba* L. explants, has enabled the modeling of explants metabolism so that one shall be able to carry out both the induction and the accomplishment of hyper-hydration. As one already knows from the renowned specialized literature, the aforementioned stand for the premises for the pre-initiation of callogenesis and, implicitly, the getting of callus.

B. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the induction and generation of callus

B.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine on *Ginkgo biloba* L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 4), which show the inter-dependence between the treatment by 6-BAP and the type of explants, for callus materialization purposes. Inter-dependence which is also shown by the callus diameter.

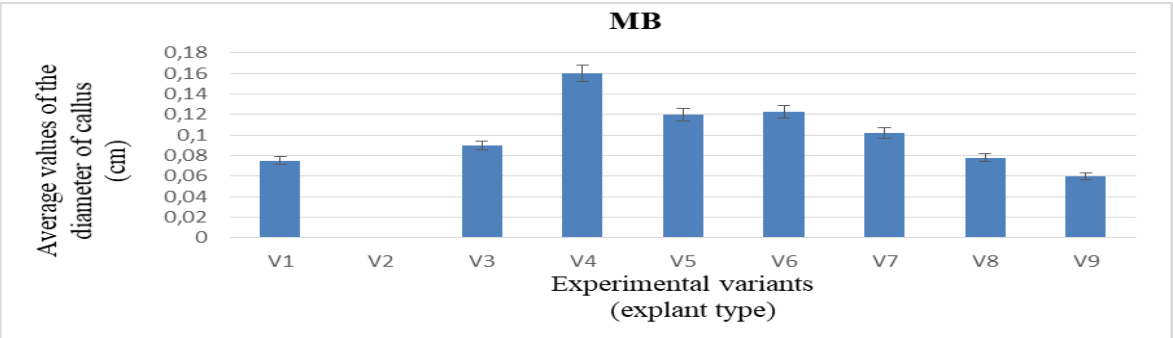

Figure 4. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine, subject to experimental variants

Given the experimental results displayed in Figure 4 it follows that the experimental variant MBV4, which consisted of the treatment by 6-BAP and of explants of the leaf type taken from the position 3 in the rosette, has enabled the occurrence of the highest average value of the diameter of callus. And the variant MBV9, which included both the treatment by 6-BAP and explants of the apical leaf section type taken from the position 7 in the rosette, has enabled the occurrence of the lowest average value of the diameter of callus. The recorded experimental results have also shown in case of the experimental variant MBV2, that there was a negative influence exercised by the treatment by 6-BAP on the explants of the leaf type taken from the position 1 in the rosette. Explants under the experimental variant MBV2 have shown senescence symptoms and they have been taken out of the experiment.

B.2. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthalene-acetic acid on *Ginkgo biloba* L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 5), which show, just like in the previously described case, the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus.

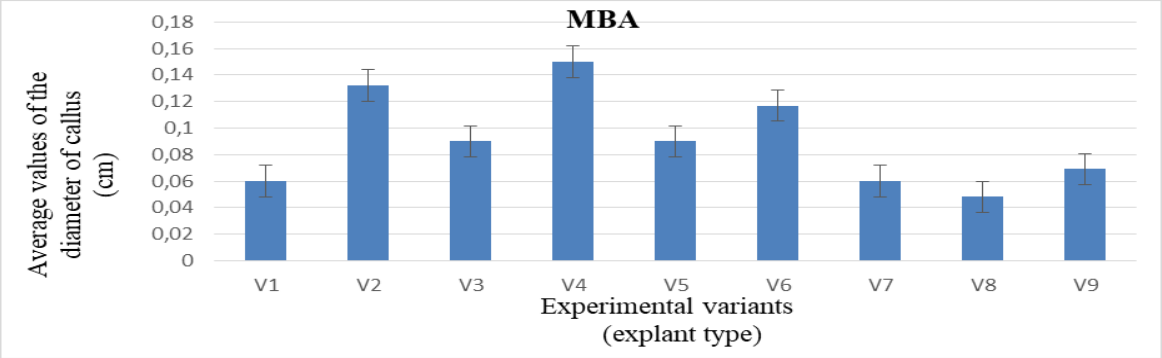

Figure 5. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine and *α-naphthaleneacetic acid*, subject to experimental variants

Based upon the experimental results shown in Figure 5 can see that the experimental variant $MBAV_4$, which has included the treatment by 6-BAP and NAA and explants of the leaf type taken from the position 3 in the rosette, has triggered the highest average value of the diameter of callus. Whereas, the experimental variant $MBAV_8$, which consisted of the treatment by 6-BAP and NAA and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of callus.

B.3. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on Ginkgo biloba L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 6), which shown, just like in the previously described two cases, the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus. Yet, just like in the case of the treatment by 6-BAP, and unlike the treatment by 6-BAP and NAA, all the nine types of explants have induced and generated callus.

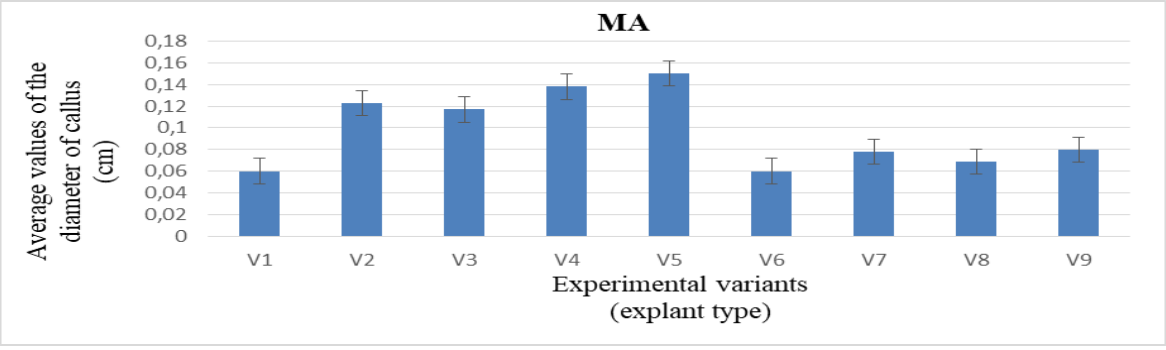

Figure 6. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 6 can see that the experimental variant MAV5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of callus. Whereas both the experimental variant MAV1 (which consisted of the treatment by NAA and explants of the type of apical bud plus a part of the stem), and the experimental variantMAV6 (which consisted of the same treatment, yet applied to explants of the leaf type with detached margins taken from the position 5 in the rosette), have registered the lowest average value of the callus diameter.

C. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the callus development

C.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine on Ginkgo biloba L. explants for callus development, has enabled the recording of quantified experimental results by determination of the diameter of the callus, only in terms of the two experimental variants. Namely, the experimental variant MBV3 (which consisted of the treatment by 6-BAP and explants of the leaf type taken over from the position 2 in the rosette), which has had the highest average value of the callus diameter (0.078 ± 0.004 cm), and the experimental variant MBV1 (which consisted of the treatment by 6-BAP and explants of the type of apical bud plus a part of the stem) which has had the lowest average value of the callus diameter (0.100 ± 0.005 cm).

Explants cultivated under the other experimental variants (MBV2, MBV4, MBV5, MBV6, MBV7, MBV8 and MBV9), have shown senescence symptoms and have been taken out of the experiment.

C.2. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthaleneacetic acid on Ginkgo biloba L. explants for callus development, has enabled the recording of experimental results (Figure 7), which show the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus.

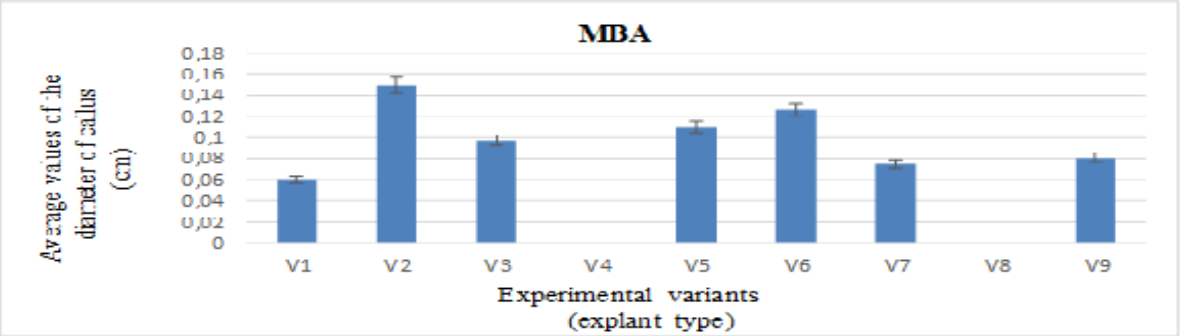

Figure 7. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 7 can see that the experimental variant $MBAV_2$, which has included the treatment by 6-BAP and NAA, and explants of the leaf type taken from the position 1 in the rosette, has triggered the highest average value of the diameter of callus. Whereas, the experimental variant MBAV1, which included both the treatment by 6-BAP and NAA, and explants of the type of apical bud plus a part of the stem, has triggered the lowest average value of the diameter of callus. At the same time, the experimental variants MBAV4 and MBAV8 have not developed callus given the contamination with microorganisms of the fungus type. Both experimental variants that have been contaminated have been taken out of the experiment.

C.3. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on *Ginkgo biloba* L. explants for callus development, has enabled the recording of experimental results which, just like in the case of the other two previously described treatments, support the idea of the existence a certain inter-dependence between the treatment and the relevant type of explants, for the purpose of materializing the callus (Figure 8).

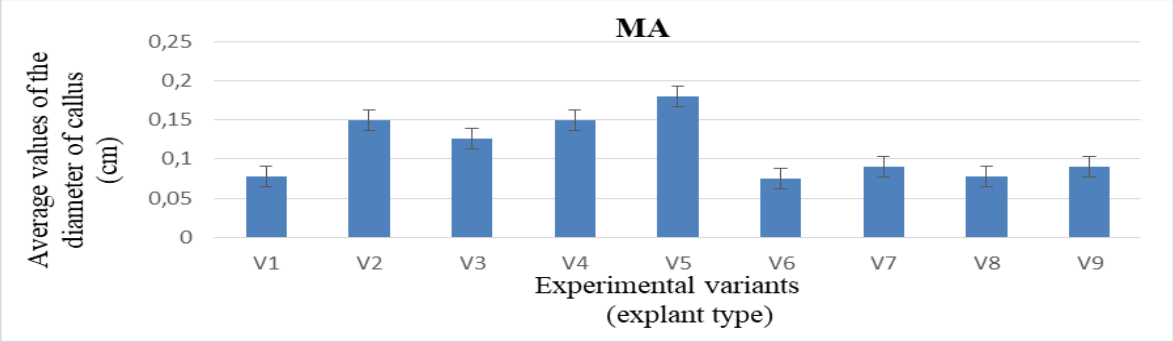

Figure 8. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 8 can see that the experimental variant MAV_5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of callus.

Whereas, the experimental variant MAV6, which consisted of the treatment by NAA and explants of the type of leaf type taken from the position 5 in the rosette, has triggered the lowest average value of the diameter of callus.

D. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the purpose of maintaining callus development

D.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthaleneacetic acid on Ginkgo biloba L. explants for the purpose of maintaining callus development, has enabled the recording of experimental results (Figure 9), which shape up the inter-dependence between the treatment and the type of explants, in view of maintaining such callus development.

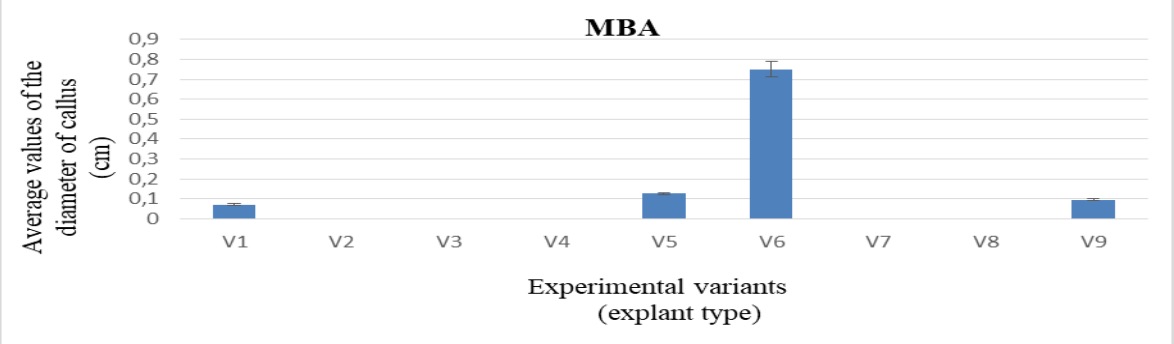

Figure 9. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 9 can see that the experimental variant MBAV6, which has included the treatment by 6-BAP and NAA, and explants of the type of leaf type taken from the position 5 in the rosette, has triggered the highest average value of the diameter of callus for the maintaining callus development.

And the variant MBAV1, which included both the treatment by 6-BAP and NAA, and explants of the type of apical bud plus a part of the stem, has enabled the occurrence of the lowest average value of the diameter of callus. Whereas the other experimental variants (MBAV2, MBAV3, MBAV4, MBAV7 and MBAV8), have been taken out of the experiment, given that they showed contaminations of the fungus type.

D.2. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on *Ginkgo biloba* L. explants for the purpose of *maintaining callus development*, has enabled the recording of experimental results quantified by the determination of the callus diameter. The results in terms of callus development (Figure 10), show the inter-dependence between the treatment and the type of explants.

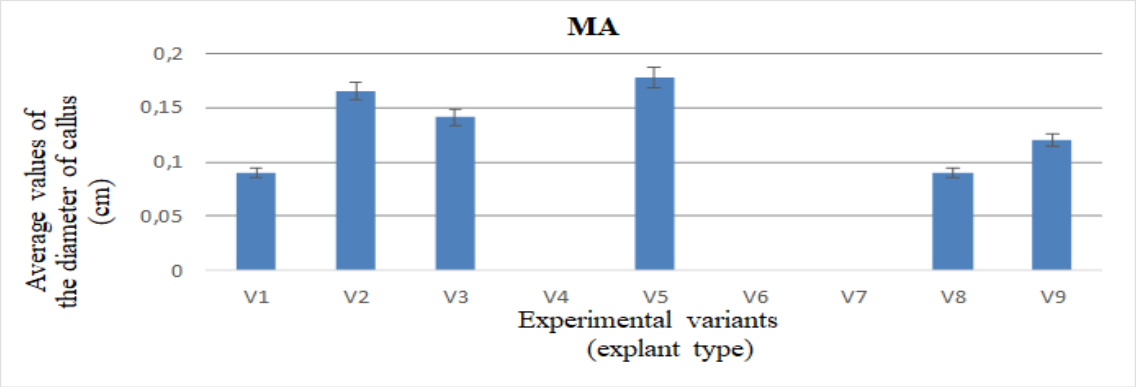

Figure 10. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

The experimental results shown in Figure 10 point out the fact that the experimental variant MAV5, namely the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has recorded the highest average value of the callus diameter for the purpose of maintaining the callus development.

Whereas both the experimental variant MAV1 (which consisted of the treatment by NAA and explants of the type of apical bud and a part of the stem) and the experimental variant MAV8 (which consisted of the same treatment, however applied to explants of the apical leaf type taken from the position 7 in the rosette), have registered the lowest average value of the callus diameter. Since three of the experimental variants (MAV4, MAV6 and MAV7), showed some contamination of the fungus type, the same have been taken out of the experiment.

By reviewing the experimental results duly achieved in terms of the **pre-initiation of callogenesis, the induction and generation of callus, callus development and maintaining such callus development**, one may notice that the treatment by α-naphthaleneacetic acid has exercised the greatest influence on *Ginkgo biloba* L. explants in view of getting callus.

Conclusions

The treatment by 6-benzylaminopurine has had the best influence on the type of explants of the leaf type taken from the position 1 in the rosette (MBV2) as related to the pre-initiation of callagenesis in *Ginkgo biloba* L.

The treatment by 6-benzylaminopurine and α-naphthaleneacetic acid has exercised the best influence on the explants of the following type: leaf taken from the position 1 in the rosette (MBAV2) for the pre-initiation of callagenesis, leaf taken from the position 3 in the rosette (MBAV4) for the induction and generation of callus, leaf taken from the position 1 in the rosette (MBAV2) for callus development and leaf with detached margins taken from the position 5 in the rosette (MBAV6) for the purpose of maintaining callus development in *Ginkgo biloba* L.

The treatment by α-naphthaleneacetic acid has exercised the best influence on the explants of the leaf type taken from the position 4 in the rosette (MAV5) both for the pre-initiation of callagenesis, the induction and development of callus, callus development and for the maintenance of callus development in *Ginkgo biloba* L.

The best influence in terms of getting callus from *Ginkgo biloba* L. explants has been achieved in the case of the experimental variant that consisted of the combination of the treatment by α-naphthaleneacetic acid and the explants of the leaf type taken from position 4 in the rosette (MAV5).

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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