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## Research Article

# Complex of Glycyrrhizic and Salicylic Acids: A New Root Length Growing Means for Grapes to Grow from the Apical Meristem

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## Abstract

**Background and Objective:** A supramolecular complex of glycyrrhizic and salicylic acids has been established as one of the potential plant growth promoters. In this study, the impact of the complex on the root growth of grapes, grown from the apical meristem was studied. **Materials and Methods:** The 1-2 mm conical meristems were isolated from the terminal and lateral buds and kept in special containers. The obtained tissues were cleaned from excessive parts and sterilized in special conditions. Further apical meristems were rooted in modified MS and WPM nutrient media. The effects of the complex on the number and length of roots of four grape varieties, widely grown in Uzbekistan, showed its potential as a root growth regulator. **Results:** The complex in 0.15 and 0.17 mg L<sup>-1</sup> doses together with benzyl amino purine in MS medium or with meta-topolin and naphthalene acetic acid in WPN culture media led to several-fold increases in root growth in all grape varieties *in vitro*. The effects of the complex at 0.15 mg L<sup>-1</sup> dose resulted in at least twice the longer root length of all selected grape varieties compared to the control of both culture media. A higher 0.17 mg L<sup>-1</sup> dose led to several-fold increases in root length. Besides, significant increases were observed in root numbers in explants, grown in a culture medium holding 0.17 mg L<sup>-1</sup> of the complex. **Conclusion:** These results could determine a new discipline in plant hormones utilized to grow plants from the apical meristem.

**Key words:** Grape varieties, plant hormone, apical meristem, supramolecular complex, *in vitro* growth, shoot ontogeny, dry mass, photosynthesis

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Apical meristem is a set of cells that divide rapidly and thus cause faster growth. Apical meristem cells at the shoot<sup>1</sup> and roots<sup>2</sup> are used to grow plants faster than generate stems during the shoot ontogeny<sup>3</sup>. These cells currently deserve high attention to develop uninfected plants. The growth from apical meristem requires a suitable nutrient medium containing plant growth-regulating hormones.

N6-benzyl adenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were reported efficient to produce axillary and adventitious shoots in seedlings. The best combination of these phytohormones was found among different options<sup>4</sup>. When N6-benzylaminopurine (BAP) and indole acetic acid (IAA) was used in different concentrations, shoot growth and meristem development were successfully achieved<sup>5</sup>. Yan *et al.*,<sup>6</sup> demonstrated the efficiency of naphthalene acetic acid on the adventitious root development of *Hemarthria compressa*, which significantly increased rooting percentage and dry mass. Approximately 15% higher rooting percentage and almost twice more dry root weight were observed in cuttings of whip grass following soaking in 100 mg L<sup>-1</sup> concentration of NAA for 30 min. In another work, BAP was shown to be efficient with MS medium plants grown with this hormone were successfully transferred to soil<sup>7</sup>. Indole-3-butyric acid (IBA) and BAP were informed as another tandem that significantly contributes to the growth of grapes *in vitro*. Their combination with MS nutrient medium resulted in the fastest development of callus<sup>8</sup>. The mixture of BAP-IBA-gibberellic acid (GA3) in a ratio of 1:0.1:0.3 with MS medium was reported as the optimum choice to grow apical meristem of *Momordica charantia* L.<sup>9</sup>. Another efficient plant cytokine 6-(3-hydroxy benzyl amino) purine, widely known by the trivial name meta-topolin, was shown to be a better choice over BAP to grow micropropagation of *Opuntia stricta* Haw in MS medium<sup>10</sup>.

Growing media and plant growth hormones often differ in functions depending on plants and even genotypes. For shooting and multiplying, MS medium containing BAP is expected an efficient growing means. MS medium, enriched with indole-3-butyric acid (IBA), is efficiently used for rooting *Vitis vinifera* L.<sup>11</sup>. A 1 0.2 mg L<sup>-1</sup> dose of IAA in MS medium resulted in the highest level of rooting of the deGrasset genotype, 2.03 roots per shoot. BAP at 1.0 mg L<sup>-1</sup> dose led to the highest proliferation level that reached 4.75 new explants on average. The tandem of IBA and NAA was found to develop callus formation of grape rootstock<sup>12</sup>. In another work, 1.5 mg L<sup>-1</sup> BAP and the combination of 1.5 mg L<sup>-1</sup> BAP and 1.0 mg L<sup>-1</sup> IBA in MS culture media were determined as the

most efficient shooting options for Soltanin and Sohebi cultivars that resulted in 3.8-5.4 shoots per grown apex. The MS media containing TDZ (1 mg L<sup>-1</sup>) and GA (1.5 mg L<sup>-1</sup>) was subsequently used as an efficient subculture medium<sup>13</sup>. The tandem of 1.0 mg L<sup>-1</sup> BAP and 0.01 mg L<sup>-1</sup> NAA in MS showed a high shooting rate for four grape cultivars that resulted in 4.69-5.50 shoots per explant. Similar effects were observed in shoot length. A 1.0 mg L<sup>-1</sup> dose of IBA led to optimum root formation that differed from 70-100% in four cultivars<sup>14</sup>.

Different growing media are commonly tested to find optimum conditions for different genotypes. In one work, Argentinian and European genotypes of *Vitis vinifera* cultivars, that originated from Europe, were tested and their *in vitro* salt tolerances were evaluated. Argentinian cultivars showed higher salt tolerance<sup>15</sup> which explains that salt tolerance is another factor affecting *in vitro* growth<sup>16</sup> reported that conditions including temperature, light and gas exchange also significantly contribute to plant growth of *Vitis vinifera* as an additional contributing factor to medium composition. The significance of these factors was linked with photosynthesis. BAP and kinetin were found inefficient in woody plant medium (WPM) to Rabbiteye blueberry micro-shoots. Zeatin, another cytokine derived from adenine, showed better responses than these two cytokines<sup>17</sup>.

These results make it necessary to develop optimum growth media for genotypes possessing different physiological responses. Improvement of the efficiency of nutrient media for plant growth from apical meristems will enable the process to use *in vitro* growth on larger scales. In this work, we inform about the modified MS and WPM nutrient media that increased the root growth of four grape varieties several-fold and enabled twice reduce ingredients of WPM nutrient medium. The complex of glycyrrhizic and salicylic acids (Fig. 1) was earlier obtained<sup>18</sup> and found to possess functions of phytohormones<sup>19</sup>.

In this study, the efficiency of phytohormone that supports the rooting of grapes in MS and WPM culture media in combination with other phytohormones was studied.

## MATERIALS AND METHODS

**Grape varieties:** Grape varieties were obtained from the existing resource centre of the Research Institute of Horticulture, Viticulture and Winemaking named after acad. M. Mirzaev, located in the Tashkent Region. Seedlings were obtained from the garden of the Scientific Research Institute of Horticulture and Viticulture named after M. Mirzaev, located in the Samarkand District. Further, the specialized collection of varieties was created in the Center of Genomics and

Table 1: Chemical composition of MS nutrient medium

Components	Dose (mg L <sup>-1</sup> )		
	Control	M <sub>1</sub>	M <sub>2</sub>
NH <sub>4</sub> NO <sub>3</sub>	1650.0	412.5	412.5
KNO <sub>3</sub>	1900.0	950.0	950.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370.0	185.0	185.0
CaCl <sub>2</sub>	331.0	165.0	165.0
KH <sub>2</sub> PO <sub>4</sub>	170.0	85.0	85.0
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	170.0	85.0	85.0
Myo-inositol	75.0	37.5	37.5
EDTA Na <sub>2</sub>	37.3	18.65	18.65
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	13.9	13.9
MnSO <sub>4</sub> ·5H <sub>2</sub> O	24.1	12.05	12.05
Glycine	10.0	5.0	5.0
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	4.3	4.3
H <sub>3</sub> BO <sub>3</sub>	6.2	3.1	3.1
Thiamine HCl	5.0	2.5	2.5
Pyridoxine HCl	5.0	2.5	2.5
Nicotinic acid	5.0	2.5	2.5
KJ	0.830	0.415	0.415
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.250	0.125	0.125
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.0125	0.0125
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.0125	0.0125
Sucrose	30.0	15.0	15.0
Agar-agar	8000.0	8000.0	8000.0
*BAP	0.50	0.25	0.25
*Supramolecular complex	-	0.15	0.17

\*Ingredients differing in control and modified nutrient media

Table 2: Chemical composition of WPM nutrient medium

Components	Dose (mg L <sup>-1</sup> )		
	Control	M <sub>3</sub>	M <sub>4</sub>
K <sub>2</sub> SO <sub>4</sub>	990.0	990.0	990.0
NH <sub>4</sub> NO <sub>3</sub>	400.0	100.0	100.0
Ca(NO <sub>3</sub> ) <sub>2</sub>	368.0	368.0	368.0
MgSO <sub>4</sub>	180.7	180.7	180.7
KH <sub>2</sub> PO <sub>4</sub>	170.0	170.0	170.0
Myo-inositol	100.0	100.0	100.0
CaCl <sub>2</sub>	72.5	72.5	72.5
EDTA Na <sub>2</sub>	37.3	37.3	37.3
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	27.8	27.8
MnSO <sub>4</sub> ·H <sub>2</sub> O	22.3	22.3	22.3
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	8.6	8.6
H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	6.2
Glycine	2.0	2.0	2.0
Thiamine HCl	1.0	1.0	1.0
Nicotinic acid	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.25	0.25	0.25
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.25	0.25
Sucrose	--	5.0	10.0
Agar-agar	8000.0	8000.0	8000.0
*Meta-topolin	--	0.48	0.48
*Naphthaleneacetic acid	--	0.1	0.1
*Supramolecular complex	--	0.15	0.17

\*Ingredients differing in control and modified nutrient media

were selected for *in vitro* growing. Grape varieties were planted at a 2.5×3 scheme.

**Sterilization process:** The explants were freed from excess tissues and kept under running water for 10 min. Sterilization of explants was performed in a laminar box (BSC 1300 IIA2-X, Jinan Biobase Biotech, China) by rinsing with 80% ethanol (5 sec), 4% sodium hypochlorite (10 min) and three times with sterilized water. The sterilized explants were filtered and prepared for planting. The apical meristems of selected cultivar samples were collected in special containers. Sterile explants were taken to Petri dishes and their apical meristems were isolated under the microscope XSP 500SM. (Ningbo Shinea Imp. and Exp. Co. Ltd., China).

**Nutrient media:** Two nutrient media-MS and WPM were used to grow explants. These nutrient media, containing sucrose, were kept in an autoclave for 20 min under 15 kg cm<sup>-2</sup> pressure and 121 °C. Further, phytohormones were added to sterilized nutrient media. The chemical compositions of the used nutrient media were given in Table 1 and 2 which was determined as M<sub>1</sub>, M<sub>2</sub> for MS and M<sub>3</sub>, M<sub>4</sub> for WPM, respectively. In these experiments, 30 explants were used to calculate every mean value.

Optimum concentrations of phytohormones were found among various concentrations: 0.40-0.55, 0.05-0.15 and 0.15-0.20 mg L<sup>-1</sup> concentration ranges were used to establish optimal doses of meta-topolin, naphthaleneacetic acid and supramolecular complexes, respectively. The supramolecular complex of glycyrrhizic and salicylic acids was added to the culture media after other plant hormones and the prepared solutions were consequently sterilized.

**Growing process:** The apical meristems were taken from grape samples starting from the second half of March till the end of April. Explants were first kept in the MS and WPM culture media for 25-28 days. Further, their cultural media were changed and kept for 28-30 days. Explants possessing roots were then held in new culture media for 21-23 days. Consequently, the culture media were changed twice for the next 21-23 days. All growing processes were carried out in a special laminar box under sterile conditions. The room temperature made 22-24 °C and the light level was 5000-6000 lux. Light and dark periods made 16 and 8 hrs, respectively. The humidity level was 72-74%.

**Statistical analysis:** Statistical analyses were performed using NSCC-2022 software. Significant differences were calculated using Tukey-Kramer's Test.

Bioinformatics of Uzbekistan Academy of Sciences for fourteen months from January, 2021 to April, 2022. From the collection, Oqdum, Rizamat, Toyfi and Rkatsiteli varieties

## RESULTS AND DISCUSSION

Glycyrrhizic acid was shown to form supramolecular complexes with salicylic acid<sup>18</sup>. Under treatment with methyl jasmonate plant hormone, it was established to elevate in the roots as one of the major components<sup>19</sup>. The complex of glycyrrhizic and salicylic acids was found to stimulate plant growth. The complex of these acids in a 1:1 ratio (Fig. 1a-d) was able to induce in the cotton plant the enzymic activity of antioxidant enzymes such as catalase, peroxidase and superoxide dismutase at very low concentrations ( $10^{-7}$  M). Besides, it stimulated plant growth under salt stress<sup>18</sup>. Glycyrrhizic acid was found to be accumulated in plant roots by abscisic acid and was correlated with another root active components<sup>20</sup>.

Salicylic acid and its derivatives were informed to reduce the level of diseases in two grape cultivars caused by *Botrytis cinerea*. Grapes, inoculated with their spores at the time of harvest, showed no symptoms if pretreated with salicylates at preharvest time<sup>21</sup>. In another work, salicylic acid treatment was shown to increase the activities of antioxidant enzymes such as ascorbate peroxidase, peroxidase, catalase and superoxide dismutase. The combination of salicylic acid with chitosan and polyvinyl alcohol was concluded to efficiently improve the storability of 'Thompson Seedless grapes<sup>22</sup>. The complex of glycyrrhizic and salicylic acids in combination with other growth regulators resulted in several-fold increases in root number (Fig. 2a-b).

Both nutrient media, holding  $0.15 \text{ mg L}^{-1}$  supramolecular complex preparation, insignificantly increased the number of

roots in all varieties-Oqdum, Toyfi, Rizamat and Rkatsiteli compared to the control. A higher concentration of the complex,  $0.17 \text{ mg L}^{-1}$ , together with other plant hormones in MS and WPM media was found to lead to several-fold increases. In Oqdum and Rizamat varieties, the number of roots at least doubled when grown in MS medium holding  $0.15$  and  $0.17 \text{ mg L}^{-1}$  concentration increased this parameter three-fold in these varieties. In Toyfi and Rkatsiteli varieties, these differences made lower level-2.3-2.5 fold differences, on average. It should be noted that both MS and WPM nutrient media resulted in very similar mean values in their controls (Fig. 3a-b).

The effects of plant hormones on the root length were more obvious than the root number. All  $M_1$ ,  $M_2$ ,  $M_3$  and  $M_4$  variants led to significant improvements in root length in all varieties when grown with both MS and WPM nutrient media (Fig. 3b). At least two-fold longer root lengths were observed when grown in nutrient media holding  $0.15 \text{ mg L}^{-1}$  of the supramolecular complex with other plant hormones. As observed with the effects of the modified MS and WPM media on root number, Oqdum and Rizamat varieties demonstrated higher effects on root length than those observed in Toyfi and Rkatsiteli varieties. The root length of these explants grown in modified MS and WPM media made almost four-fold mean values compared to their controls. This improvement in Oqdum and Rizamat varieties made  $>5$ -fold (Fig. 2). It should be noted that the overall image of variants in MS and WPM media were similar and differences were observed only in varieties.

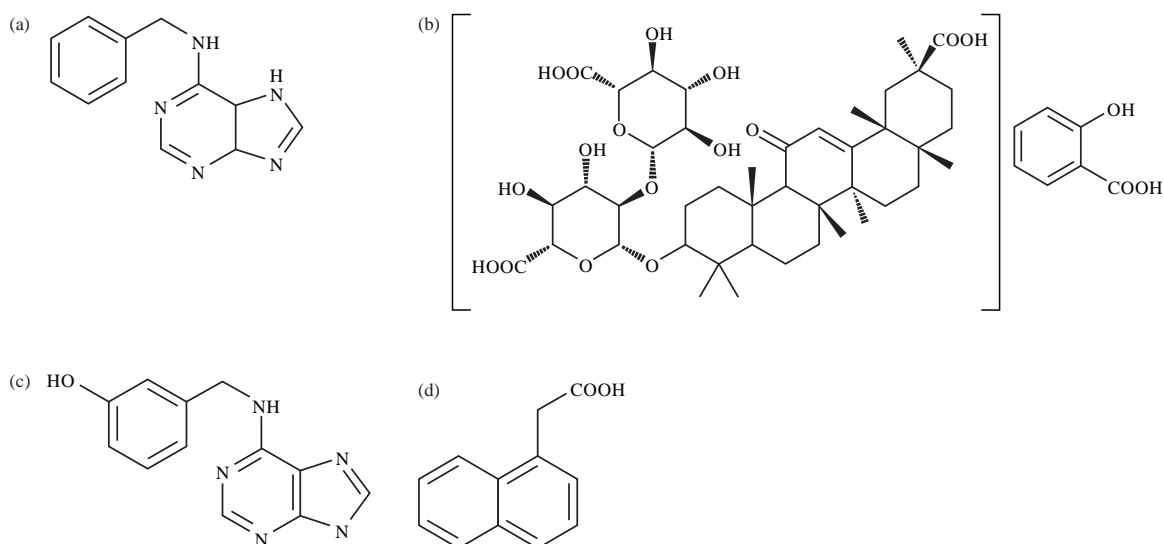


Fig. 1 (a-d): Chemical structures of plant hormones used in this study, (a) 6-Benzyl amino purine, (b) Supramolecular complex of glycyrrhizic and salicylic acids, (c) Meta-topolin and (d) Naphthaleneacetic acid

\*Structures were drawn in ChemDraw Prime (19 version) and further cleared up

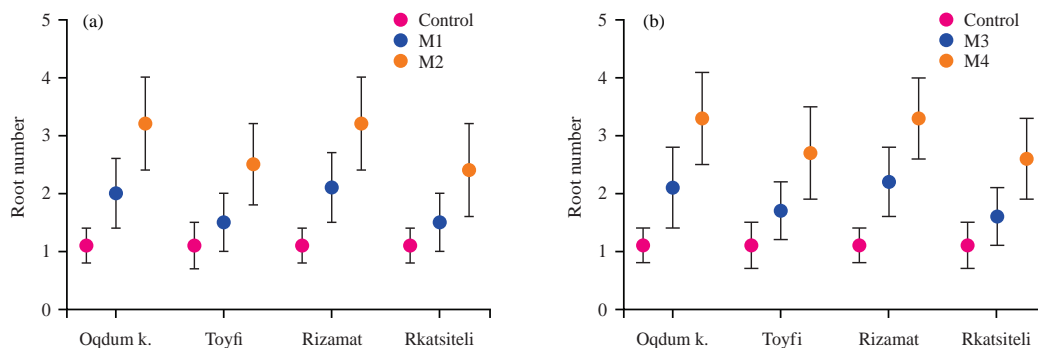


Fig. 2(a-b): Effects of modified nutrient media on the root numbers of grape varieties grown *in vitro*, (a) Modified MS nutrient medium and (b) Modified WPM nutrient medium

\*Error bar Means ± SD and X-axis: Grape varieties

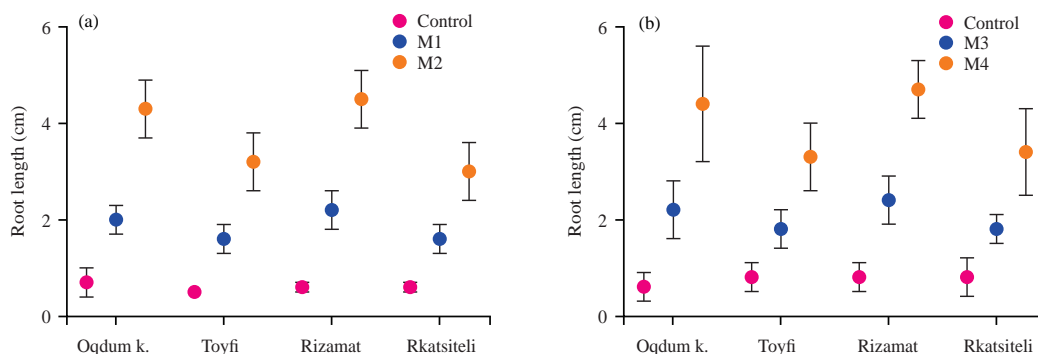


Fig. 3(a-b): Effects of modified nutrient media on the root length of grape varieties grown *in vitro*, (a) Modified MS nutrient medium and (b) Modified WPM nutrient medium

\*Error bar Means ± SD and X-axis: Grape varieties

Table 3: All-pairs comparisons of p-values (root number)

Comparisons	Root number		Root length	
	M <sub>2</sub> medium	M <sub>4</sub> medium	M <sub>2</sub> medium	M <sub>4</sub> medium
Oqdum-Rkatsiteli	0.0000 (Y)	0.0001 (Y)	0.0000 (Y)	0.0004 (Y)
Oqdum-Rizamat	0.9856 (N)	0.9862 (N)	0.5540 (N)	0.7064 (N)
Oqdum-Toyfi	0.0009 (Y)	0.0019 (Y)	0.0000 (Y)	0.0001 (Y)
Rkatsiteli-Rizamat	0.0000 (Y)	0.0000 (Y)	0.0000 (Y)	0.0000 (Y)
Rkatsiteli-Toyfi	0.8332 (N)	0.8331 (N)	0.9294 (N)	0.9534 (N)
Rizamat-Toyfi	0.0006 (Y)	0.0012 (Y)	0.0000 (Y)	0.0000 (Y)

Multiple comparison type: Tukey-Kramer. Hypotheses tested: H<sub>0</sub>: Diff = 0 vs H<sub>1</sub>: Diff ≠ 0. Y: Yes, N: No rejection of H<sub>0</sub> at 5.0%. Rejection decisions are based on adjusted p-values that are computed using the number of comparisons and the adjustment type

In this study, similar effects of the modified nutrient media on root number and length of explants of four grape varieties were observed. Insignificant differences were calculated in Oqdum-Rizamat and Rkatsiteli-Toyfi tandems (Table 3).

The addition of meta-topolin, naphthaleneacetic acid and supramolecular complex in 0.5:1:0.17 mg L<sup>-1</sup> doses, respectively, increased the assimilation of ammonium nitrate

in WPM nutrient media and enabled to reduce of its quantity in the medium four-fold. But the addition of 10 mg of sucrose was required for the revealed efficiency of the modified MS nutrient medium. These differences in WPM nutrient media were even greater. The addition of 0.17 mg L<sup>-1</sup> supramolecular complex of glycyrrhizic and salicylic acids and 0.25 mg L<sup>-1</sup> of benzylaminopurine resulted in several-fold effects with the twice-lower quantity of all used ingredients.

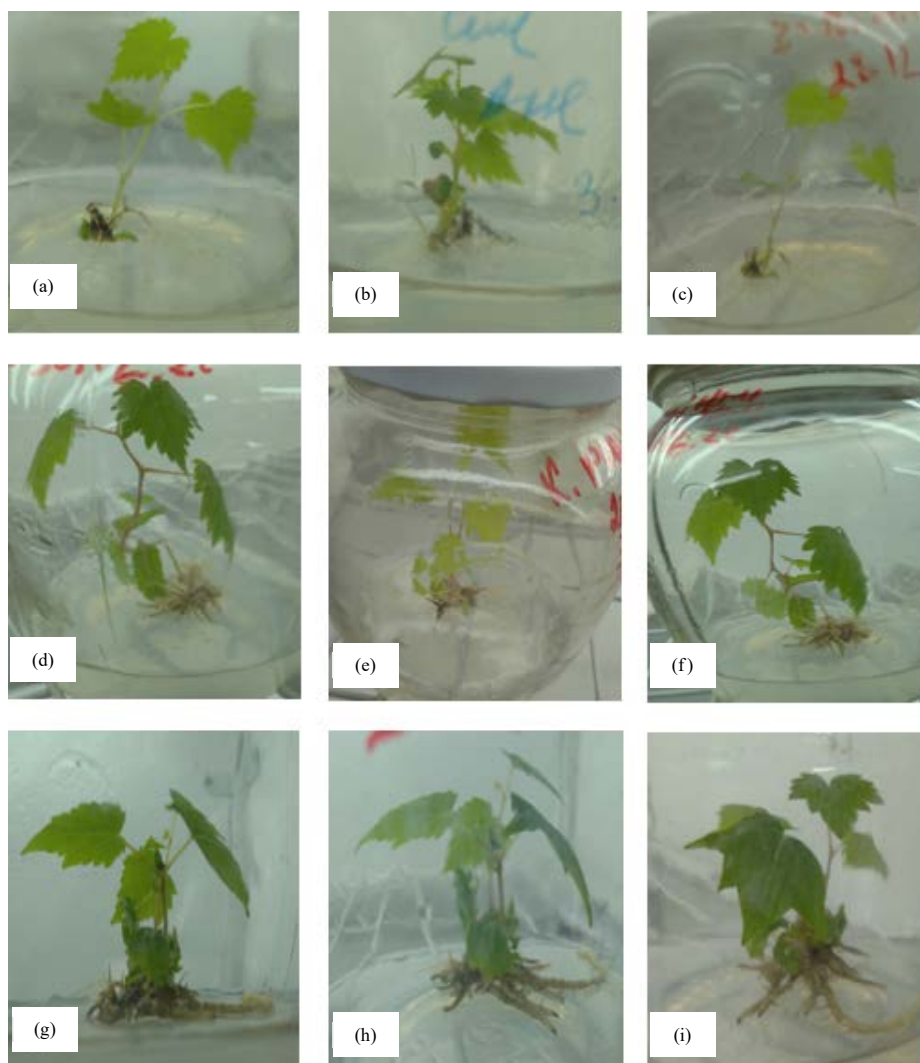


Fig.4(a-i): Improvement of root length in Rizamat variety following the treatment with MS nutrient medium, (a-c) Control, (d-f)  $M_1$  medium and (g-i)  $M_2$  medium

The highest effects of the modified nutrient media were observed with those explants taken in May and April. The ones, taken in August and September, did not reveal efficiency with both MS and WPM nutrient media. In December, January and February, the plants growing in nutrient media stopped growing and the appearance of new shootings started in March and April.

Besides the root length, the treatment with the supramolecular complex of glycyrrhizic and salicylic acid preparation with other plant hormones resulted in strengthened roots. Remarkable differences in the root thickness were observed (Fig. 4a-i). In control plants grown with unmodified MS and WPM nutrient media, the root colour turned light black which evidences the slackened root

development. Moreover, the modified nutrient media  $M_2$  and  $M_4$  led to remarkable differences in the colour of the leaves compared with the unmodified control.

The supramolecular complex of glycyrrhizic and salicylic acids is a new plant growth-promoting agent established in our laboratory for grapes and pomegranate (unpublished data). Since the complex has not become popular and has not reached the market yet, comparing its effects on grape varieties with other literature is limited to separate components. Salicylic acid has been established to promote *in vitro* plant growth in *Vanilla planifolia* Jacks<sup>23</sup>. It was found to support *in vitro* growth of *Hibiscus moscheutos* under salt stress<sup>24</sup>. It was also reported as an agent attenuating the stress by abiotic and biotic factors including salinity, heavy metals or

pathogens<sup>25</sup>. These results prove the effect of salicylic acid included in the complex in this study. An enhanced level of glycyrrhizin by jasmonic acid derivative was determined to inhibit root growth of *Glycyrrhiza glabra*. However, glycyrrhizin levelled up by salicylic acid did not interfere with root growth<sup>26</sup>. Besides high amounts of glycyrrhizic acid in the roots of *Glycyrrhiza glabra*, grown under drought stress, agreed with higher root diameter in a two-year study<sup>27</sup>. The grape varieties, studied in this work, are widely grown in Uzbekistan. Among these varieties, Rizamat is the most widely spread one which is grown in Central Asia and the Xinjiang region. It is one of the most used varieties to develop new genotypes<sup>28</sup>. As a seeded grape variety, it contains recessive loci for seedlessness<sup>29</sup>. Alternate drip irrigation of the Rizamat variety was shown to keep the same photosynthesis level but a lower transpiration rate<sup>30</sup>. Alternate partial-root-zone irrigation was further shown to improve water usage based on evapotranspiration. The new approach led to increased content of soluble solid content<sup>31</sup>. The Rizamat variety is expected susceptible to *Erysiphe necator* powdery mildew<sup>32</sup>.

The Rkatsiteli cultivar was shown to accumulate the majority of metals in concentrations lower than the control. Plasma optical emission spectroscopy revealed higher concentrations of copper and arsenic<sup>33</sup>. The pomace of the Rkatsiteli variety was suggested to be an attractive resource of tocopherol and other antioxidants that could be used in the pharmaceutical and food industry<sup>34</sup>.

Toyfi is one of the well-known grape varieties in Uzbekistan that is grown in climatic regions of the republic<sup>35</sup>.

Growing grapes *in vitro* enable to obtain plants not subjected to infection. From this point of view, this approach is preferred even if it costs more expensive. Cost-effectiveness can be achieved by reducing the quantities of used ingredients as well as plant hormones. These obtained results will enable us to further increase the efficacy of the modified MS and WPM media in terms of the reduction of used ingredients by improving their assimilation via plant hormones and the new supramolecular complex. Thus, the cost-effectiveness of the modified nutrient media will enhance.

## CONCLUSION

In this study, the effectiveness of 0.17 mg L<sup>-1</sup> of the supramolecular complex of glycyrrhizic and salicylic acids with 0.25 mg L<sup>-1</sup> BAP in MS medium and with 0.48 mg L<sup>-1</sup> meta-topolin and 0.1 mg L<sup>-1</sup> naphthaleneacetic acids in WPM nutrient media were reported. No significant differences were

observed in the efficacy of the modified MS and WPM media in our case. Further improvement in this discipline will enable the enhancement of the cost-efficiency of the modified nutrient media.

## SIGNIFICANCE STATEMENT

Various hormones are used in growth nutrient media. Due to their high cost, their utilization is limited to research. In this work, we used a supramolecular complex of glycyrrhizic and salicylic acids and established its high efficiency to grow grapes *in vitro* from the apical meristem. It has been demonstrated to grow apical meristem greatly in 0.17 mg L<sup>-1</sup> dose in combination with 0.25 mg L<sup>-1</sup> BAP.

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