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Influence of micropropagation with addition of kinetin on development of a willow (*Salix viminalis* L.)

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ABSTRACT

The aim of the dissertation was to determine (select) the best growth medium for a basket willow (*Salix viminalis* L.) which contains optimal concentration of kinetin. The plant material taken from three varieties of willows: Bjor, Jorr and Tora, which grew in the experimental field, was sterilized and placed on a complete MS (Murashige and Skoog, 1962) growth medium. The propagated plants were used in an experiment, where MS substrate was enriched by growth regulator – kinetin, in various concentrations (0.5 $mg \cdot dm^{-3}$, 1 $mg \cdot dm^{-3}$, 2 $mg \cdot dm^{-3}$ and 3 $mg \cdot dm^{-3}$). The substrate which contained 0.5 KIN was considered to be the best for willow's propagation, because plants cultivated with usage of this medium have shown the largest mass, the highest number of shoots and leaves, and also the highest quantitative and elongation growth of the roots. Among examined varieties of *Salix*, the best one was Bjor variety, where evaluated parameters were distinctive in comparison to two remaining varieties.

***Keywords*:** micropropagation, cytokinin, *Salicaceae*, MS medium

1. INTRODUCTION

Belonging to the family of *Salicaceae*, *Salix* variety comprises about 450 species of deciduous trees and shrubs located all over the world, mainly in the Northern Hemisphere.

Due to a large number of varieties and clones and common hybridization between species it is difficult to deliver a precise number of species (Grönroos, 1995, Naujoks, 2007). Willows, in general, are found in temperate and arctic zones, however, at present they are also possible to be found in subtropical and tropical zones. Geographical distribution of willows embraces almost all of the continents, with exception of Australia and Antarctica (Kuzovkina i Quigley, 2005, Sharawy, 2016).

Salix are the trees and shrubs mainly found to be growing on damp and wet soils. Willows are found in lowland meadow habitats, shores of rivers, dunes, riparian forests along flowing water, and also swamps and mountain areas (Naujoks, 2007, Sharawy, 2016). Almost all of the species of *Salix* variety are dioecious, with numerous entomophilous flowers gathered in cylindrical inflorescences called aments (Grönroos, 1995).

In beneficial conditions trees of many species of willow become in most of cases pioneers of vegetative and generative propagation. Willows play an important role in a development of particular structures of a landscape and in maintaining ecological balance (Naujoks, 2007). Smart and Cameron (2008) claim, that willows are pioneers among species, which tolerate shade and are able to colonize various kinds of places, also in riparian and swamp habitats. Besides that, they are a perfect for plantations used to acquire bioenergy. Many species of the *Salix* family are characterized by a high ability to adapt to physiological conditions. That is the reason why economic significance of a basket willow (*Salix viminalis*) in recent years is rising, e.g. to restore destroyed ecosystems, including phytoremediation and bioengineering (Kuzovkina i Quigley, 2005). Also at present willows are used to reduce noise, as walls protecting shores of lakes, in wickerwork industry, as a support for beekeeping and also in purification of municipal sludge, recycling of communal sewage and to produce energy (Grönroos, 1995). Willows are also used as resource for bioenergy and pharmaceutical goods production, or as nourishing filters, because of their high growth potential (Naujoks, 2007). Propagation in *in vitro* conditions may be an alternative for such plant group. However, micropropagation of plants from *Salix* family is still technically difficult (von Aderkas i Bonga, 2000).

Willow (Salix) is showing anti-bacterial, anti-viral, anti-parasite and also anti-fungal activities. Moreover, some of willow's species, due to their wide-spread root system and quick growth are often used in phytoremediation of contaminated soil (Perttu i Kowalik, 1997, Vervaeke et al., 2003). Willows may survive and develop in hydroponic system, even with presence of heavy metals and temperature exceeding normal, critical concentrations (Punshon i Dickinson, 1997).

According to Mashkina et al. (2009) *Salix* is one of the most dynamically developing tree species. Willow plantations are created in order to fulfill needs, among others, in paper industry, wicker trading, to extract tannin and production of nourishing additions and to plant greenery. Plants of many species of willow and hybrids are evaluated as potentially perspective objects for bioenergy plantations, which will be used to produce liquid or gas fuel, such as ethanol, butanol and biogas. High rate of growth and possibility of vegetative propagation, adapting to various soils and climate conditions and also large specific variability, cause that *Salix viminalis* is a species, which provides highly perspective biomass production.

Most of the species of willow is easily propagated through seedlings, but techniques of tissue culture are interesting while taking into account clonal propagation of species or clones where developing roots might prove difficult. Micropropagation also allows to speed up many

culture programs, at the same time reducing risk of spreading diseases in international exchange of plant material. These kinds of techniques may also become valuable tools to create and detect plant strains having desired features, such as resistance to diseases or frost tolerance. There had been many researches related to tissue culture of ligneous plants, however values which relate to Salix species, are considerably rare, despite the fact that willows were one of the first plants cultivated in *in vitro* conditions (Bergman et al., 1985).

Mohamed et al. (2015) and Sharawy (2016) claim, that tissue culture is a method of maintaining and propagate plant tissue in artificial *in vitro* environment in stable, strictly controlled conditions. Propagation of plants in *in vitro* conditions became an important and at the same time popular method, because stable supply of sterile seedlings prevailed over the problem of plant contamination and reduced the time of sterilization process. However, there are many factors influencing the ability of tissue to create callus, they include regulators of plant's growth. Besides, scientist consider that regeneration of shoots from callus may cause somaclonal variability, however direct regeneration of shoots from leaves or parent transplants may eliminate such undesired effects. In process of plant propagation from adventitious or axillary buds, *in vitro* is a useful technique for producing clonal seedlings. There are some reports about propagating various species of willow from axillary shoots, however, because shoots grow out of initially formed buds, there are only two publicized researches related to *in vitro* regeneration of *Salix* species from lateral buds or somatic embryos.

As Naujoks (2007) claims micropropagation is a tool which facilitates propagation of chosen genotypes of a willow, which previously were characterized by outstanding results, such as growth, disease resistance, and also perfect quality of wooden elements. Usually *Salix* species are propagated in *in vitro* conditions only through apical meristem, buds, shoots or node segments with a lateral bud. Explants are commonly cultivated with usage of modified mediums (Murashige and Skooga) MS (Murashige and Skoog, 1962) or WPM (Lloyd i McCown, 1980) supplemented by growth regulators (auxin and cytokinin) in order to trigger growth of whole plant (shoot and root). However, common problems in *in vitro* cultures are contaminations and hyperhydration which may lead to a death of an explant. Survival of explants depends in a high degree on physiological stadium of plant it was derived from and from a season, in which exhibits were taken to create a cultivation. However, previously developed methods for particular species appeared to be relatively dependent on a genotype. It was proved however, that somatic embryogenesis is possible, moreover, *Salix* may become a perfect model system for genetic research. Willows are plants, which may be easily propagated vegetatively with usage of seedlings, without significant problems related to aging of plants. They have a small genome and short generation time, about 2 years and they already became a subject of many cultivation programs (Grönroos, 1995, Palomo-Ríos et al., 2015, Skálova et al., 2012).

Paiva Neto et al. (1998) claim, that efficient usage of tissue culture technique in propagation of plants is related most of all to usage of growth regulators, cultivation in aseptic conditions and usage of smaller quantity of plant material to start a cultivation, what enabled easier spreading of various ligneous species. The aim of hereby experiment was determining the best growth medium for a basket willow (*Salix viminalis* L.) containing optimal concentration of kinetin.

2. MATERIAL AND METHODS

Biological material used to start hereby experiment was constituted by a basket willow (*Salix viminalis* L.) in three varieties: Bjor, Jorr and Tora, which was taken from plants growing in experimental field prepared by Department of Plant Physiology and Biochemistry (West Pomeranian University of Technology in Szczecin). On the other hand starting material was derived from mother plantation Hvidsted Energy Forest in Denmark. Acquired plant material was constituted by cuttings from the youngest, one year old shoots.

The experiment was performed in 2016 in Department of Genetics, Plant Breeding and Biotechnology of the West Pomeranian University of Technology in Szczecin. Plant material was acquired in February, after formation of buds, but before formation of leaves. Because of that fact, gathered plants for period of two weeks were placed in warm room in a container with distilled water, up to the moment of lateral shoots formation (Pict. 1). Then, the material was sterilized.

Photograph 1. Willow cuttings after formation of shoots from lateral buds.

Plant material was cut to smaller fragments in order to obtain explants, which was carefully flushed for 10 minutes with running water with addition of detergent (washing-up liquid – Ludwik). Afterwards the plants were submerged for 15 seconds in 70% alcohol, then they were put for 5 minutes into 10% sodium hypochlorite. Then explants were flushed three times in sterile distilled water for 5 minutes. Next, each of the explants, constituting a node with bud was placed in a separate test-tube on a basic culture medium (Pict. 2), which is MS

medium (Murashige and Skoog, 1962), without plant growth regulators. After two weeks explants which developed rooting were transferred to jars, in which they grew for another three weeks. This way acquired sterile plants were used in the experiment.

Photograph 2. Following stages of growth and propagation of explants (a, b and c), basing on example of Bjor variety.

Experiment was performed in the Department of Genetics, Plant Breeding and Biotechnology from propagated plants of a basket willow (*Salix viminalis* L.) in three varieties: Bjor, Jorr and Tora. MS (Murashige and Skoog, 1962) was used as a culture medium, which contained various quantity of macro and microelements. Controlling variant was a complete MS medium, and experimental variants were enriched by kinetin (KIN) with concentration: 0.5 $mg \cdot dm^{-3}$, 1 $mg \cdot dm^{-3}$, 2 $mg \cdot dm^{-3}$ and 3 $mg \cdot dm^{-3}$. The medium also contained 8 $g \cdot dm^{-3}$ of agar, 30 $g \cdot dm^{-3}$ of sucrose and 100 $mg \cdot dm^{-3}$ of inositol. The pH of the medium was determined and modified to 5.7 from 0.1 of M NaOH and HCI. Then 30 ml doses were poured into 300 ml jars and sterilized in autoclave for 20 minutes in temperature equal to 121 °C. The plants were growing in climate chambers with 16 hour photoperiod (16 h with light / 8 hours without light), in temperature equal to 24 ± 1 °C. Shelves in climate chambers were illuminated with fluorescent light with power of 36 W, generating light with 35 mmol $m^{-2}s^{-1}$ intensity. Each of the three varieties of the willow in each experimental combination and in control sample was created in 8 repetitions, and each jar contained five plants. Experiment lasted for eight weeks, and after this period, biometrical measures were taken. Following parameters were evaluated: height of the plants [cm], shoots quantity, leaves quantity, length of roots [cm], quantity of roots and plant's weight [g]. The results of measures were subjected to statistical analysis. Significance of differences was determined by applying Tukey's test with $p = 0.05$.

3. RESULTS AND DISCUSSION

Acquiring aseptic cultures of ligneous plants, including willow with high morphogenetic activity and life span is one of the most complex stages of culture in *in vitro* conditions. Moreover, creating tissue cultures is connected with a high degree of bacterial or fungal infections. Initial material which is subjected to sterilization, often has reduced or completely lost regenerative activity of tissues, what leads to their aging (Mashkina et al., 2010).

Willows growing on a appropriate culture media have an ability to quickly spread roots and grow long shoots which have many leaves. Therefore, it is possible, to propagate plants quickly through repetitive division of stem tissue, what will lead to larger number of node segments (Chalupa, 1983).

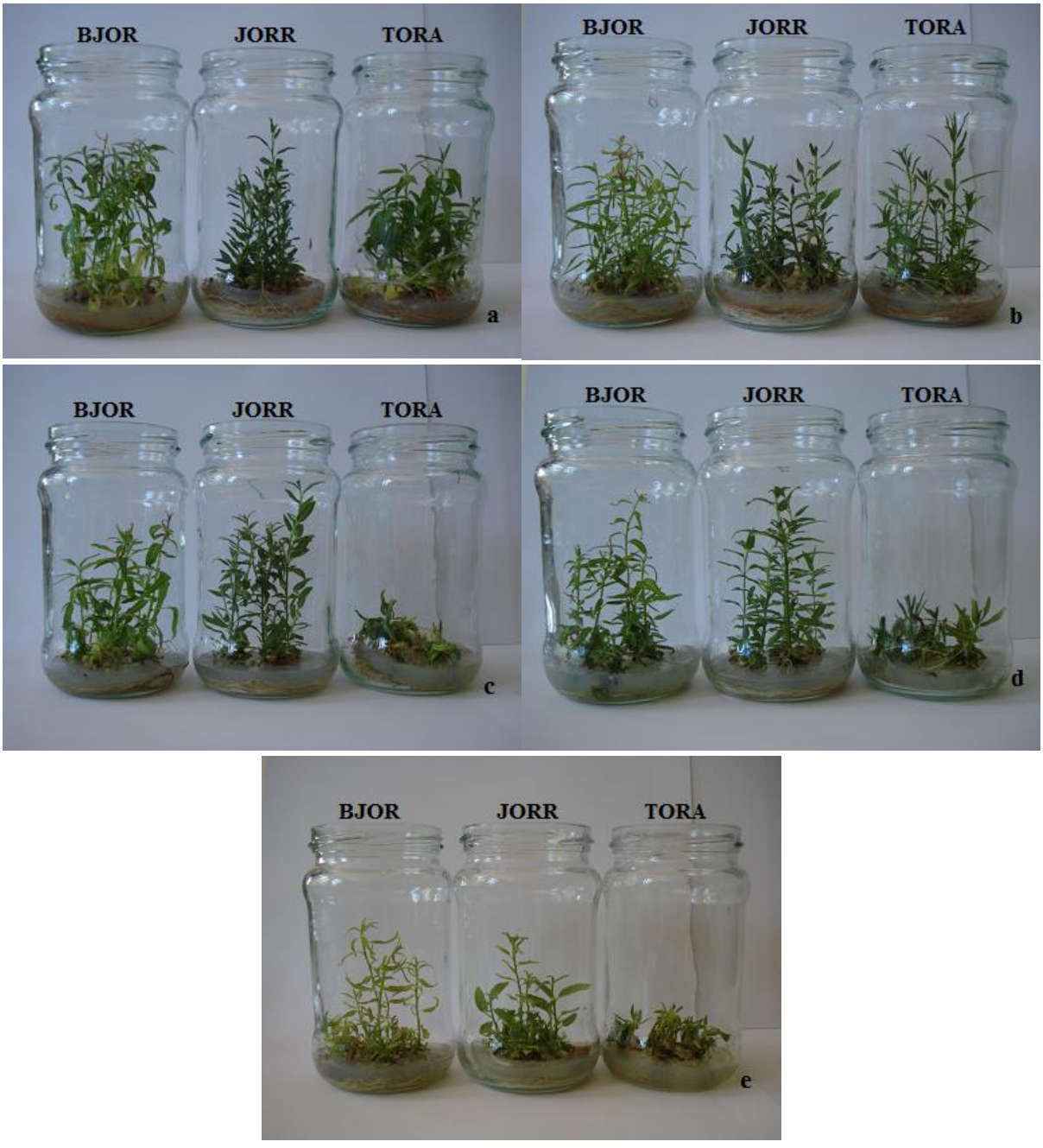
According to Khan et al. (2011) cytokinin is necessary in case of *in vitro* propagation of plants from lateral buds of roots and proliferation. However, efficient and optimal concentration is different for each species of a plant. Cytokinin plays a key role in DNA synthesis and division of cells, what may become a stimulator for induction of many shoots. Furthermore, differentiated reaction of plants which we observe is connected to various levels of concentration of various types of cytokinin.

Experiment was conducted on sterile plants of a basket willow (*Salix viminalis* L.) in three varieties: Bjor, Jorr and Tora (Pict. 3) on MS medium. In many experiments conducted in *in vitro* cultures with many species of willow (*Salix* L.) MS medium was used. This medium was used to conduct research in regard to many species of *Salix*, including *S. tarraconensis* (Amo-Marco i Lledo, 1996), *S. alba, S. fragalis, S. triandra, S. viminalis* (Chalupa, 1983, Grönroos 1995, Mala et al., 2010), *S. babylonica* (Dhir et al., 1984, Palomo-Ríos et al., 2015), *S. lapponum* (Pogorzelec et al., 2015), *S. safsaf* (Mohamed et al. 2015). Equally popular growth medium is WPM, which was used in many researches by such scientists as: Amo-Marco and Lledo (1996) for *S. tarraconensis,* Liesebach i Naujoks (2004) and Naujoks (2007) on *S. caprea*. Researches for *S. viminalis* (Bergman, 1985), *S. tarraconensis* (Amo-Marco i Lledo, 1996), *S. nigra* (Lyyra et al., 2006), *S. humboldtiana* (Paiva Neto et al. 1998), *S. pseudolasiogyne* (Park et al., 2008) were also conducted. In all mentioned researches, as well as in hereby research, the sources of explants were cuttings, shoots with lateral buds or leaves. Most of the researchers added to a standard medium (MS or WPM) such growth regulators as: KIN, BAP, IAA, IBA, NAA, BA, GA3, in various concentrations.

In performed experiment, similar regulators were used. In all of the experimental combinations, an answer and reaction was formation of shoots, roots and leaves, however in some of cases callus was formed. Such reaction was recorded in their research. Many researchers conducted their experiments on various species of Salix, including a basket willow, but they were not performed with usage of MS medium containing various KIN concentrations (Bhojwani, 1980, Lyyra et al., 2006).

Culture medium was constituted by complete MS medium (Murashige and Skoog, 1962), enriched in experimental variants by various kinetin concentrations (KIN). Complete MS medium was a control variant, where height of plants [cm] in each of examined varieties of willows were the highest (Table 1). Among varieties growing on MS medium the largest height of plants was observed in Bjor clone (7.2 cm). Among mediums enriched by kinetin (KIN) the highest plants were determined to grow with the lowest concentrations of cytokinin

(0.5 KIN) in Jorr variety (5.6 cm) and Tora (4.5 cm), on the other hand Bjor 1 KIN (6.2 cm). Between varieties, there had been determined significant statistical differences as it comes to height of plants growing on various mediums, these differences were also determined for mediums (Tab. 1).



Photograph 3. Comparison of three varieties (Bjor, Jorr and Tora) *Salix v.* L. in MS (a) control medium and experimental 0.5 KIN (b), 1 KIN (c), 2 KIN (d), 3 KIN (e).

Table 1. Height of the plants [cm] of three varieties of willows on mediums with different kinetin concentration.

Content of kinetin [mg·dm^{-3}]	Variety			Average
	Bjor	Jorr	Tora	
MS (control)	7,2	6,7	5,4	6,4
0,5 KIN	5,7	5,6	4,5	5,3
1 KIN	6,2	5,5	2,2	4,6
2 KIN	5,0	5,5	3,6	4,7
3 KIN	5,4	4,2	2,4	4,0
Average	5,9	5,5	3,6	
Variety			0,97	
Medium			0,96	
Interaction V x M			r. n.	
Interaction M x V			r. n.	

Statistical analysis of number of shoots formed in particular varieties in dependence on a kind of medium did not show significant differences for interaction, despite the fact that its number was ranging from 1.6 for Jorr on MS medium, up to 3.6 for Bjor on medium with addition of 0.5 KIN (Tab. 2). Statistically significant differences were observed only between examined varieties, for average results. Bjor variety formed the largest number of shoots, significantly larger than Jorr variety.

Table 2. Number of shoots among three varieties of a willow growing on mediums with various concentration of kinetin.

Content of kinetin [mg·dm^{-3}]	Variety			Average
	Bjor	Jorr	Tora	
MS (control)	2,3	1,6	2,4	2,1
0,5 KIN	3,3	2,8	3,6	3,2
1 KIN	3,3	2,3	2,5	2,7
2 KIN	3,0	2,7	3,3	3,0
3 KIN	3,3	2,7	2,1	2,7

Average	3,0	2,4	2,8	
Variety			0,52	
Medium			r. n.	
Interaction V x M			r. n.	
Interaction M x V			r. n.	

Research related to the number of leaves among examined varieties of the basket willow, in dependence on medium did not show significant differences, as well for interaction as for main effects, this is varieties and kind of mediums (Tab. 3)

Table 3. Number of leaves among three varieties of a willow growing on mediums with various concentration of kinetin.

Content of kinetin [mg·dm^{-3}]	**Variety**			**Average**
	Bjor	**Jorr**	**Tora**	
MS (control)	28,8	31,2	25,7	28,5
0,5 KIN	39,6	34,9	31,7	35,4
1 KIN	34,7	32,4	19,9	29,0
2 KIN	30,7	33,1	31,6	31,8
3 KIN	32,1	30,1	28,6	30,3
Average	33,2	32,3	27,5	
Variety			r. n.	
Medium			r. n.	
Interaction V x M			r. n.	
Interaction M x V			r. n.	

Used mediums were differentiating the length of the roots among particular varieties of a basket willow, however, statistically they were insignificant (Tab. 4). Significant differences were determined for mediums and varieties. Among mediums the one which was stimulating spreading the roots in a best way, was medium with addition of 0.5 KIN, where the longest roots were determined. In case of varieties, Bjor was clearly characterized by the longest roots, and Tora by the shortest roots, respectively 14.0 and 8.4 cm.

Table 4. The length of roots [cm] among three varieties of a willow growing on mediums with various concentration of kinetin

Content of kinetin [mg·dm^{-3}]	Variety			Average
	Bjor	Jorr	Tora	
MS (control)	14,9	8,6	12,5	12,0
0,5 KIN	17,9	14,9	10,9	14,6
1 KIN	16,1	13,5	6,3	11,9
2 KIN	12,2	10,4	6,5	9,7
3 KIN	9,0	5,6	5,7	6,7
Average	14,0	10,6	8,4	
Variety	2,72			
Medium	2,68			
Interaction V x M	r. n.			
Interaction M x V	r. n.			

Results related to the number of roots are presented in table number 5. Significant differences were only determined for kinds of mediums, independently of variety. The highest number of roots was noted in MS control medium and in the one with 0.5 KIN addition, respectively 10.2 and 10.3.

Table 5. Number of roots among three varieties of a willow growing on mediums with various concentration of kinetin.

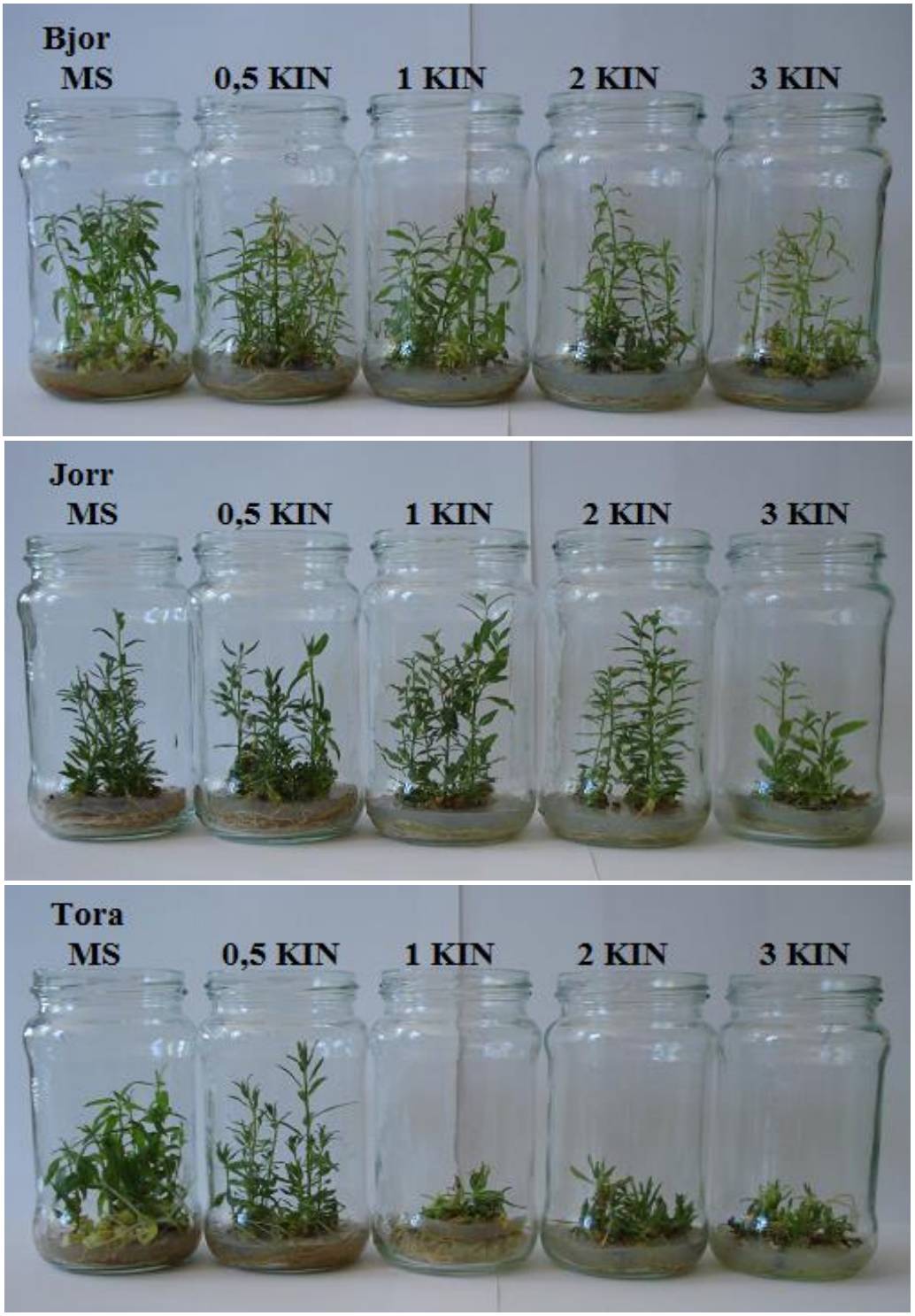
Content of kinetin [mg·dm^{-3}]	Variety			Average
	Bjor	Jorr	Tora	
MS (control)	10,4	7,8	12,3	10,2
0,5 KIN	8,3	11,2	11,4	10,3
1 KIN	7,4	8,5	7,2	7,7
2 KIN	6,2	7,1	7,6	7,0
3 KIN	6,3	6,0	6,4	6,2

Average	7,7	8,1	8,9	
Variety			r. n.	
Medium			1,90	
Interaction V x M			r. n.	
Interaction M x V			r. n.	

Measured weight of plants (Tab. 6) in each experimental combination was varying, and was statistically insignificant, despite the fact that among all of the varieties the largest weight was noted for 0.5 KIN medium (Tab. 6). Significant statistical differences were determined only for the main effect, this is the mediums. Similar as in the case of majority of examined parameters, the best medium – which stimulated rise of weight was MS medium with addition of 0.5 KIN.

Table 6. Plant's weight [g] among three varieties of a willow growing on mediums with various concentration of kinetin

Content of kinetin [mg·dm^{-3}]	**Variety**			**Average**
	Bjor	**Jorr**	**Tora**	
MS (control)	0,986	0,839	1,177	1,000
0,5 KIN	1,114	1,362	1,238	1,238
1 KIN	0,943	0,955	0,608	0,836
2 KIN	0,687	0,795	0,711	0,731
3 KIN	0,728	0,811	0,490	0,676
Average	0,892	0,952	0,845	
Variety			r. n.	
Medium			0,255	
Interaction V x M			r. n.	
Interaction M x V			r. n.	



Photograph 4. *Salix v.* L. in three varieties: Bjorr, Jorr and Tora in all concentrations of medium in experimental combinations and control medium.

The results indicate that the most advantageous for the growth of *Salix viminalis* L. is a medium containing 0.5 mg·dm^{-3} KIN. Plants cultivated on this medium were characterized by the largest number of shoots (3.2), and consequently the largest number of leaves (35.4), they also had better developed roots and were one of the highest among all experimental combinations (Picture 4).

The results acquired in the research allowed to measure the best growth medium for *Salix viminalis* L. containing kinetin in the optimal concentration, which does not hinder development of plants. It may allow to propagate willow in *in vitro* cultures in an efficient way, and in future it may allow to protect endangered species.

4. CONCLUSIONS

1) The best growth medium for *Salix viminalis* L. is MS enriched by 0.5 mg·dm^{-3} of kinetin (KIN), on which examined varieties of willow acquired the most advantageous parameters, the largest height, number of shoots, leaves and roots; length of roots, plant weight.
2) Culture medium containing over 0.5 mg·dm^{-3} of kinetin was slowing down the growth of plants and changed their growth habit.

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