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MORPHOLOGICAL CHANGES IN *NICOTIANA TABACUM* TYPE PRILEP, VARIETY P 12-2/1, INFECTED WITH FRESH *AGROBACTERIUM RHIZOGENES* A4 CULTURE IN *IN VITRO* CONDITIONS

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ABSTRACT

The aim of this paper is to show that infection of tobacco (*Nicotiana tabacum*) with a fresh culture of *Agrobacterium rhizogenes* A4, under *in vitro* conditions, lead to genetic transformation. The interaction between the t-DNA sequence of Ri plasmid of *Agrobacterium rhizogenes* A4 and recipient cells of *Nicotiana tabacum* seed type Prilep variety P 12-2/1, induces the appearance of "Hairy Roots-HR" and positive morphological modifications are manifested by increased surface area, number of leaves and lateral roots, and increased length of root and offspring. The recent research assume that these transgenic plants with altered phenotype can be very usefull in future in industry for production of biofuel.

Keywords: *Agrobacterium rhizogenes*, tobacco (*Nicotiana tabacum L.*), morphological modifications, "Hairy Roots-HR"

МОРФОЛОШКИ ПРОМЕНИ КАЈ *NICOTIANA TABACUM* ТИП ПРИЛЕП, СОРТА П 12-2/1 ПРИ ИНФЕКЦИЈА СО СВЕЖА КУЛТУРА ОД *AGROBACTERIUM RHIZOGENES* А4 ВО *IN VITRO* УСЛОВИ

АПСТРАКТ

Овој труд имаше за цел да покаже дека при инфекција на тутун (*Nicotiana tabacum*) во *in vitro* услови со свежа култура од *Agrobacterium rhizogenes* А4, настанува генетска трансформација. Интеракцијата помеѓу t-DNA секвенцата од Ri плазмидот на *Agrobacterium rhizogenes* и клетката реципиент на семето од *Nicotiana tabacum* тип Прилеп сорта П 12-2/1 индуцира појава на "Hairy Roots-HR"-"влакнести корени" и позитивни морфолошки модификации кои се манифестираат со зголемена површина и број на листови и странични корени, како и зголемена должина на изданок и корен. Најновите истражувања претпоставуваат дека овие трансгенични растенија со променет фенотип во иднина можат да бидат од голема корист во индустријата за производство на биогориво.

Клучни зборови: *Agrobacterium rhizogenes*, тутун (*Nicotiana tabacum L.*), морфолошки модификации, "Hairy Roots-HR"-"влакнести корени".

INTRODUCTION

In the eighties of the last century first attempts were made to use *Agrobacterium rhizogenes* A4 as a vector for introduction of foreign genes of interest in the plant genome (Horch et al., 1985). Cultures of hairy roots (HR) are obtained by integration of the t-DNA fragment of Ri plasmid of *Agrobacterium rhizogenes* into the plant cell genome. Then, the place of infection of the plant tissue induced development of transgenic hairy roots (Chilton et al., 1982). During the transformation process with *Agrobacterium rhizogenes* one or two t-DNA sequences were transferred into the plant genome (Jouanin, 1984). With integration of T_L t-DNA sequence in the genome of the plant cell allows the expression of *rol* genes that have essential role in induction, development of the phenotype and HR (Binns and Costantino, 1998). On the other hand, the integration of T_R t-DNA sequence is enabling expression of genes responsible for the biosynthesis of auxin in the plant cell genome, with the increased content of auxin, and the need for exogenous phytohormones for development and growth transformed HR decreases. Also with transmission of T_R t-DNA segment of the Ri plasmid of *Agrobacterium rhizogenes* into the plant cell genome occurs expression of genes for the biosynthesis of specific opini (Rhodes et al., 1990). Cultures of the HR have the capability of spontaneous regeneration in transgenic plants that are characterized by expression of active t-DNA genes (Tepfer, 1984). The regenerated transgenic plants have modified morphological characteristics in comparison to non-transformed plants, a newly phenotype known as "hairy root phenotype" or "T - phenotype". This phenotype is characterized by curved leaves, reduced plate surface, the emergence of asymmetrical and colorful leaves, highly developed root system, reduced apical dominance, reduced length of internodes, etc (Tepfer, 1984; Hamamoto et al., 1990). Growing of crops of genetically transformed roots were characterized by two physiological parameters (Kuzovkina and Schneider, 2006): linear elongation of the root tip and exponentially formation of lateral roots. The curve growth is characterized by a short resting phase (lag phase), the following exponential increase of the biomass of the HR culture. The cell cycle of HR cultures is 2-7 days (Wilson et al., 1987). The exponential growth phase of HR ends by forming a dense combination of intertwined fibrous roots. During the growing HR limiting the necessary growth factors that restrict the space and reduced supply of oxygen and nutrients from the nutrient medium. Also cultures of HR have the ability to form idioblast. Idioblast does not contain chloroplasts and represent cells with thickened cell walls. The presence of lipophilic vesicles and vacuoles in idioblast are of great importance in the accumulation of secondary metabolites. The process of growing the HR is incomparably more intense than untransformed roots. HR have the ability to form new root meristems high degree of lateral branching (Oksman-Caldentey and Hilyunen, 1996).

MATERIAL AND METHODS

Determination of growth optimal conditions for *Agrobacterium rhizogenes* A4

A. rhizogenes A4 is part of Collection of Microorganisms, from Department of Microbiology and Microbial Biotechnology, Faculty of Natural Sciences and Mathematics in Skopje. The initial culture of *A. rhizogenes* A4 was inoculated on three media: Mannitol Broth, Nutrient Broth and Muller Hinton Broth, with incubation time

of 48 hours and a temperature of 28-30°C. Before and after the incubation period it was measured turbidity of bacteria in order to determine the most suitable medium for incubation. Then, in order to determine the optimum pH value of the medium, the initial culture was incubated in appropriate medium in five different pH values: 6, 6.5, 7, 7.5 and 8, during the incubation of 72 hours and a temperature of 28-30°C. After the incubation period, it was measured turbidity of bacteria in order to determine the optimum pH. Finally, *A. rhizogenes* A4 was incubated in an appropriate medium, appropriate optimal pH, at three different temperatures: 23/25◦C, 28◦C and 37◦C for 48 hours. Turbidity measurements before and after incubation were varied, whereby to determine the most appropriate temperature for incubation of bacteria.

Preparation of fresh bacterial suspension of *Agrobacterium rhizogenes A4*

Bacterial suspension of *A rhizogenes A4* was prepared in Muller Hinton broth with a pH adjusted to 6.5 with 0.1N NaOH. 3 ml of bacteria were incubated in 300 ml broth, on 28°C, during 48 h, on shaker. After two days it was obtained fresh culture for infection of seed tobacco *(Nicotiana tabacum)* type Prilep, variety P 12-2 /1.

Infection and micropropagation of *Nicotiana tabacum* type Prilep, variety P12-2 /1

One part of tobacco seed was infected with *A. rhizogenes* A4, and one part was uninfected, and utilized as control for the experiment. Infection was carried out for a period of 30 minutes. After that, seeds were placed in double-distilled water for imbibitions, than it was surface sterilized in 70% ethanol (1 minute) and 1% Izosan-G (10 minutes). After washing several times in distilled deionized water it was planted in small jars (40 ml) with ½ MS basal medium (Murashige and Skoog, 1962, Gamborg, 1968). The basal medium contains 30 g·L^{-1} sucrose, 7 g·L^{-1} agar, MS mineral solution (Murashige и Skoog, 1962, Gamborg, 1968) vitamin B5 solution, organic (Table 1) and inorganic components (Table 2). After 14 days of micropropagation, from the sterile sprouts seeds we were isolated apical buds in length of 1-3 mm, as initial explants. These explants were placed on MS medium.

Table 1. Organic components of the MS basal medium.

Organic components	Concentration (mg·L^{-1})
Thiamine (vitamin B1)	0,1
Pyridoxine (vitamin B6)	1,0
Nicotinic acid	0,1
Casein hirolizat	200,0
myo-inositol	100,0

Table 2. Inorganic components in the MS basal medium.

Inorganic components	Concentration (mg·L^{-1})
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$x $2H_2O$	441

$MgSO_4$x $7H_2O$	370
KH_2PO4	170
H_3BO_3	6,2
$MnSO_4$ x H_2O	16,9
$ZnSO_4$ x $7H_2O$	8,6
KJ	0,83
Na_2MoO_4 x $2H_2O$	0,25
$CuSO_4$ x $5H_2O$	0,25
$CoCl_2$ x $6H_2O$	0,25
Na_2EDTA	37,26
$FeSO_4$ x $7H_2O$	27,8

The cultures were kept in a climate chamber under controlled aseptic conditions. The seeds sprout successfully for a period of 14 days under *in vitro* conditions. After the 15th day it was measured the length of the sprout and the root, number of lateral roots and leaves on sprout, the length and width of each leaf, and the values were used to calculate the surface of the leaves. Eventually the plant material of experimental and control groups was lyophilized to absolutely dry condition.

RESULTS AND DISCUSSION

Determination of growth optimal conditions for *Agrobacterium rhizogenes* A4

To select the most suitable medium for incubation of *Agrobacterium rhizogenes* A4, it was tested three different media: Mannitol Broth, Nutrient Broth and Muller Hinton Broth, with the incubation time of 48 hours and a temperature of 28-30◦C. Growth was monitored by turbidity change before and after the incubation period because the turbidity is proportional to the number of bacteria in the medium. Table 3 shows that the most appropriate medium is Muller Hinton broth (1 FAU before/ 280 FAU after).

Table 3. Media for culturing bacteria.

medium	**turbidity before incubation (FAU)**	**turbidity after incubation (FAU)**
Nutrient Broth	0	156
Mannitol Broth	0	267
Muller Hinton Broth	1	280

To select the most suitable pH value for the incubation of *Agrobacterium rhizogenes* A4, bacteria was inoculated on MH medium with five different pH values (6, 6.5, 7, 7.5 and 8). According to turbidity, the most suitable medium was the one with a pH 6.5 (Table 4).

Table 4. pH values for culturing bacteria.

pH	pH turbidity before incubation (FAU)	turbidity after of incubation (FAU)
6	4	372
6,5	14	555
7	3	418
7,5	0	235
8	4	529

To choose the optimal temperature for cultivation of *A. rhizogenes* A4, bacteria were exposed to three different temperatures, 23/25°C, 28°C and 37°C for a period of 48 hours. After incubation it was obtained the results shown on Table 5.

Table 5. Temperatures for culturing bacteria.

Temperature	turbidity before incubation (FAU)	turbidity after incubation (FAU)
23/25°C	12	233
28°C	4	371
37°C	1	179

Morphological changes in *Nicotiana tabacum* type Prilep, variety p 12-2/1 infected with fresh *Agrobacterium rhizogenes* A4 culture in *in vitro* conditions

Agrobacterium rhizogenes infects higher plants to produce so-called ''hairy roots'' with altered phenotype from the wound sites. Transformed root cultures possess some properties that make them very attractive in comparison with untransformed root cultures and undifferentiated cell suspension and tissue cultures (Kuzovkina and Schneider, 2006).

In our study, one half of tobacco seeds was infected with *A. rhizogenes* A4 and one half which was not infected with bacteria was used as a control for the experiment. Sown seeds were left to germinate for a period of 14 days. After the germination of the seed and growing of tobacco plant, it was analysed morphological features of infected tobacco and control. In the experiment we included morphological characteristics as the length of the sprout and the root, number of lateral roots and leaves on sprout, the length and width of each leaf. After lyophilization, dry weight of control was 0.019g, while the treated material 0.126g.

Hairy roots are fast growing and laterally highly branched, and are able to grow in hormone-free medium. Moreover, these organs are not susceptible to geotropism anymore (Sevon and Oksman-Caldentey, 2002).

Table 6. Length of the sprout and the root, number of leaves per sprout and number of lateral roots in five control samples.

	Length of the sprout (mm)	Root length (mm)	No. of leaves per sprout	No. of lateral roots
Control 1	19	38	7	11

Control 2	18	22	7	7
Control 3	14	19	6	7
Control 4	12	31	6	6
Control 5	9	7	7	3
Average	14,4	23,4	6	7
SD	4,159327	11,84483	0,547723	2,863564
Max	19	38	7	11
Min	9	7	6	3

* Control 1-5: random samples were taken from five uninfected material; Average: arithmetic average of all obtained values for each parameter; SD: standard deviation; Max: maximum value; Min: minimum value

Table 7. Length, width and area of the leaves in five control samples.

	Length of leaf (mm)	Width of leaf (mm)	**Area of leaf (mm^2)**
C1	11	9	32
	7	5	35
	11	9	33
	6	7	42
	7	4	28
	6	3	34
	7	3	77
	11	7	74
	13	6	82
A.v, C1	8,8	5,9	48,6
C2	6	5	30
	6	5	30
	3	2	6
	4	3	12
	4	3	12
	4	2	8
	4	3	12
A.v, C2	4,4	3,2	15,7
C3	5	3	15
	5	2	10
	4	2	8
	4	3	12
	7	6	42
	7	6	42

	4	3	12
A.v, C3	5,2	3,6	20,2
C4	5	3	15
	5	3	15
	6	4	24
	8	5	40
	7	4	28
	4	3	12
A.v, C4	5,8	3,7	22,4
C5	3	2	6
	4	3	12
	2	2	4
	4	1	4
	2	2	4
	6	5	30
	2	2	4
A.v, C5	3,3	2,4	9,1
Average value	5,5	3,7	12,9

* A.v, C1-C5. mean value of control leaves ** average value of Av, C1-C5

The transformed roots can be excised to establish axenic root cultures and indefinitely propagated in growth regulator free medium. The root exhibit fast, plagiotropic growth characterized by profuse lateral branching and rapid root tip elongation, just like in several earlier papers (Tepfer and Tempé, 1981; Chilton et al., 1982; Tepfer, 1984).

Table 8. Length of the sprout and the root, number of leaves per sprout and number of lateral roots in five samples treated with *A. rhizogenes* A4

	Length of the sprout (mm)	Root length (mm)	No. of leaves per sprout	No. of lateral roots
T1	27	35	7	9
T2	56	45	9	13
T3	65	32	5	11
T4	46	52	5	16
T5	42	49	7	9
Average	47,2	42,6	7	12
SD	14,4118007	8,73498712	1,67332005	2,96647939
Max	65	52	9	16
Min	27	32	5	9

*T1-5: random samples were taken from five infected material; Average: arithmetic average of all obtained values for each parameter; SD: standard deviation; Max: maximum value; Min: minimum value

Table 9. Length, width and area of the leaves in five samples treated with *Agrobacterium rhizogenes* A4.

	Length of leaf (mm)	Width of leaf (mm)	Area of leaf (mm^2)
T1	4	3	12
	4	3	12
	16	11	176
	11	7	77
	7	4	28
	7	3	21
	8	6	48
A.v,T1	8,2	5,3	54
T2	11	9	99
	7	5	35
	11	9	99
	11	7	42
	7	4	28
	18	13	234
	19	13	247
	22	17	374
	21	12	252
A.v, T2	13,6	9,9	156,7
T3	15	11	165
	9	8	72
	7	9	63
	10	11	110
	8	9	72
A.v,T3	9,8	5,3	96,4
T4	13	8	104
	11	8	88
	12	8	96
	9	7	63
	6	5	30
A.v,T4	10,2	7,2	76,2
T5	9	6	54
	8	7	56
	9	8	72
	9	6	54
	8	5	40
	8	6	48
	5	5	25
A.v, T5	8	6,2	49,8
Average value	9,9	6,7	86,5

* A.v, T1-T5. mean value of treated leaves ** average value of average value, T1-T5.

Morphological modifications in comparison to the length of the sprout, the length of the root, the number of lateral roots, the number of leaves per sprout (Table 8) and the surface area of the leaves (Table 9) were positive and they are manifested in in-

creased growth. According to these observations, treated sprouts and roots of infected samples had a length which is 3 times higher than in control samples (Table 6 and Table 7).

The experimental data in our study showed similar behavior as results published for *V. vinifera* cultivars (Martins et al., 2003; Peros et al., 1998).

Fig1. Germinated non-infected seeds of tobacco (*Nicotiana tabacum*), type Prilep, variety P12-2 /1.

Fig2. Germinated infected seeds of tobacco (*Nicotiana tabacum*), type Prilep, variety P12-2 /1.

The number of leaves and number of lateral roots is increased by about 2 times. There was also positive morphological modifications in length, width and surface of leaves. A. rhizogenes, like A. tumefaciens, invokes morphological changes in infected plant tissues and allows growth of transformed tissues in vitro in the absence of exogenous plant growth regulators. However, rather than undifferentiated tumors, highly branched, ageotropic roots emerge from sites of A. rhizogenes infection. Transformed roots can be regenerated into plants which, in many species, have a characteristic morphology (called the “hairy root” phenotype) that includes stunted growth, shortened internodes, reduced apical dominance, severely wrinkled leaves, atypical flower morphology and reduced fertility (Tepfer, 1984).

In infected tobacco samples appeared hairy root syndrome (Hairy root) as a result of the transfer of genetic material (t-DNA) of bacteria cells in the tobacco seed (Figure2), whereas in the control samples this phenomenon was absent (Figure1).

The promising developments and applications of hairy root cultures indicate that in the near future these cultures will provide researchers with powerful tools for further biotechnological research.

CONCLUSIONS

The results of investigations lead to the following conclusions:

- Optimal conditions for growth of *Agrobacterium rhizogenes* A4 are: Muller Hinton Broth – as a medium; 6,5 - pH value; 28°C - temperature.
- In *in vitro* conditions we successfully obtained multiplied sprouts from isolated buds from tobacco (*Nicotiana tabacum*), type Prilep, variety P12-2 /1 on MS mineral medium.

As a result of the contact between the t-DNA sequences of the Ri plasmid of the bacterium and the recipient cell from tobacco seeds, the treated material was characterized by the appearance of hairy roots, while in the control material this phenomenon is absent.

The control material was characterized normal phenotype, and the treated material was altered morphology. Morphological modifications of the treated material were positive, and values were increased two to three times compared to the control.

Overall, hairy roots seem to be becoming useful in the production of proteins, in environmental biotechnology for phytoremediation of pollutants from waste water, and for the regeneration of genetically altered plants

REFERENCES

1. Binns, A. N., Costantino, P. 1998. The *Agrobacterium* Oncogenes. In: Spink, H., Kondorosi, A., Hooykaas, P. J. J. (eds). The Rhizobiaceae (pp. 251–266). Kluwer Academic Publishers, Dordrecht.
2. Chilton, M. D., Tepferd, A., Petit, A., David, C., Casse-Debart, F., Tempe, J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature. 295, 432-434.
3. Gamborg, O. L., Miller, R. A., Ojima, K. 1968. Nutrient requirements of suspension cultures soybean root cells. Exp. Cell Res. 50, 148-151.
4. Hatamoto, H., Boulter, M. E., Shirsat, A. H., Croy, E. J., Ellis, J. R. 1990. Recovery of morphologically normal transgenic tobacco from hairy roots co-transformed with *Agrobacterium rhizogenes* and a binary vector plasmid. Plant Cell Rep. 9, 88-92
5. Horsch, R., Fry, J., Hoffmann, N., Eichholtz, D., Rogers, S., Fraley, R. 1985. A simple and general method for transferring genes into plants. Science 227, 1229–1231.
6. Jouanin, L. 1984. Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmids. Plasmid 12. 91–102.
7. Kuzovkina, I. N., Schneider, B. 2006. Genetically transformed root cultures. Generation, Properties and Application in Plant Sciences, Progress in Botany. 67, 275-324.
8. Martins, T. M., Domingos, A., Novo, C., Lourenço, P. M. L.2003. Effect of *Agrobacterium rhizogenes* infection on *in vitro* rooting of *Vitis vinifera.* Vitis 42 (3), 159–161.
9. Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Phys. Plant. 15, 473-497.
10. Oksman-Caldentey, K. M., Hiltunen, R. 1996. Transgnic crops for improved pharmaceutical products. Field Crops Res. 45, 57-69.
11. Péros, J., Torregrosa, L., Berger, G. 1998. Variability Among *Vitis Vinifera* Cultivars In Micropropagation, Organogenesis And Antibiotic Sensitivity. J. Exp. Bot. 49, 171 - 179.
12. Rhodes, M. J. C., Robins, R. J., Hamill, J. D., Parr, A. J., Hilton, M. G., Walton, N. J. 1990. Properties of transformed root cultures. In: Charlwood, B. V., Rhodes, M. J. C. (eds) Secondary products from plant tissue culture. Clarendon Press, Oxford, 201–225.
13. Sevón, N., Oksman-Caldentey, K.-M. 2002. *Agrobacterium rhizogenes*-Mediated Trans-

formation: Root Cultures as a Source of Alkaloids. Planta Med 68(10), 859-868.
14. Tepfer, D. 1984. Genetic transformation of several species of higher plants by *Agrobacterium rhizogenes*: phenotypic consequences and sexual transmission of the transformed genotype and phenotype. Cell 37, 959–967.
15. Tepfer, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. Physiol. Plantarum 79, 140-146.
16. Tepfer, D., Tempé, J. 1981. Production of d'agropine par des racines transformes sous I'action *d'Agrobacterium rhizogenes* souche A4. CR Acad Sci 292, 153–156.
17. Wilson, P. D. G., Hilton, M. G., Robins, R. J., Rhodes, M. J. C. 1987. Fermentation studies of transformed root cultures. In: Bioreactors and Biotransformations. (Moody, G. W., Baker, P. B., eds.). London: Elsevier, 38-51.