Influence of activated charcoal and different concentrations of vitamins on the androgenesis of tobacco from the Krumovgrad ecotype

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Abstract
In vitro androgenesis is a technique for shortening the breeding process, but the factors of influence is many and is particularly specific to the diferent crop. This necessitated long-term experimental specification of individual components and concentrations of culture media for each specific genotype.

The effect of active charcoal (AC) and different concentrations of B - complex vitamins on the initial stages of androgenic culture of tobacco Krumovgrad ecotype, in 6 different media (with standard prepared Nitsch and Nitsch vitamins, vitamin B - complex with and without added activated charcoal (AC) in solid state) were study.

The experiment was conducted in two years with of 135 flower buds - Krumovgrad ecotype. *In vitro* growth was reported in all media, and nutrient growth rates of 1, 2, 4 and 5 are close, showing that B-complex vitamins could be used instead of standard ones. The strongest development of regenerants was reported at higher concentrations of B-complex.

The probable combination of low concentrations of vitamins with active charcoal (AC) has a favourable effect on the initial stages of development of androgenesis in tobacco plants of the Krumovgrad ecotype.

Key words: androgenesis; tobacco; culture medium; active charcoal; vitamins

INTRODUCTION

In vitro androgenesis is an important component of plant biotechnology when pollen grains pass from their normal pathway of pollen development to an embryogenic pathway. Haploid and doubled haploids produced by androgenesis have long been recognized as a valuable tool in plant breeding because they can shorten the breeding cycle, fix agronomic traits in the homozygous state, and increase the efficiency of selection for useful recessive agronomic traits (Rukmini & Jwala, 2016).

The use of the dihaploid (DH) technique also has its limitations, as the formation of haploid plants depends on many factors (Vural et al., 2019; Irikova et al., 2011). Tobacco is considered a modal organism for *in vitro* techniques and these have long been introduced and successfully used in its breeding programs. Regardless of the good androgenic potential of tobacco in general, optimization of conditions for *in vitro* androgenesis should be done for each genotype (Atanassov & Djilianov, 1997).

In tobacco androgenesis protocols, the following critical stages are considered: flower bud stage, their pre-treatment and culture media (Sood et al., 2021). Nich and Nich culture medium has been shown to be effective in tobacco androgenesis (Miceska et al., 2009). Growth and morphogenesis

of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins, amino acids and some undefined additives, such as fruit juices, coconut milk, yeast or malt extracts, and hydrolyzed casein (George et al., 2007).

Vitamins in combination with other components of culture media have direct and indirect effects on callus growth, somatic growth, rooting and embryonic development (Abrahamian & Kantharajah, 2011).

Plant cells grown *in vitro* can synthesize essential vitamins in insufficient amounts. For this reason, the culture medium is often supplemented with vitamins to increase growth. Some studies reported that the best androgenic results were obtained from MS medium supplemented with different types of B vitamins (biotin, folic acid and cobalamin) (Ozsan & Onus, 2017; Demirkaya & Comlekcioglu, 2021). Cell requirements for added vitamins vary according to the nature of the plant and the type of culture (Abrahamian & Kantharajah, 2011).

Besides vitamins, another component with an expected positive effect in culture media for androgenesis in differents cultures is activated charcoal (AC). Haploid plants of *Nicotiana tabacum* L. are considered to be produced in greater numbers from anthers on agar medium containing activated charcoal than on the same medium without charcoal (AC) (Anagnostakis, 1974).

Activated charcoal (AC) has a very fine network of pores with a large internal surface area on which many substances can be adsorbed. It is often used in tissue culture to improve cell growth and development (Thomas, 2008). Its addition to culture media can stimulate or inhibit growth *in vitro*, depending on the species and tissues used. Studies in potatoes (Buckseth, et al., 2018) found an increase in microplant height and root length compared to the control (MS without AC). In wheat, culture media containing AC promoted the growth of young plants by increasing the expression of certain genes in the phenylpropanoid biosynthesis pathway, which are related to cell differentiation and growth, as well as genes involved in plant hormone signaling, which is related with resistance (Dong et al., 2020).

The effect of charcoal can be attributed to the creation of a darkened environment; adsorption of unwanted/inhibiting substances; adsorption of growth regulators and other organic compounds or release of growth promoting substances present in or adsorbed on AC (Pan & Staden, 1998).

According to some authors, the difficulty in using AC in the cultural media is that in addition to adsorbing unwanted substances, it can adsorb necessary hormones, vitamins or metal ions such as Cu^{+2} and Zn^{+2} (Thomas, 2008). Therefore, the addition of AC to cultural media needs further research to specify concentrations to avoid negative effects (Buckseth et al., 2018).

The objective of this research was to study the effect of AC and vitamins of group B with different composition and concentration on the initial androgenesis stages of tobacco Krumovgrad ecotype-

MATERIALS AND METHODS

Materials

Flower buds of donor plants Krumovgrad ecotype were used in the experiments. A total of 135 flower buds are set (in 2020 - 75, and in 2021 – 60). All donor plants were grown at Tobacco and Tobacco Products Institute – Markovo according to agricultural technology for oriental tobacco growing.

- Culture media (CM) - six variants were prepared according to the recipe of Nitch & Nitch (1969) with added 0.001 mg/L-IAA and 1 mg/L - kinetin. Two of the culture medium variants are prepared with commercial B-complex vitamins in two concentrations. For the preparation of the stock solutions of both concentrations, 1 tablet was removed from the alginate coating and 0.0100 g (10 mg) dissolved in 100 ml dH_2O and 0.0500 g (50 mg) dissolved in 100 ml dH_2O were weighed from it respectively to provide of higher concentrations (from the prepared starting solutions of vitamins, 1 ml is taken for 1 L from the respective culture medium). 100 mg/L myo-inositol and 2 mg/L glycine were also added. Different variants of culture media are prepared with and without charcoal (AC). In the present study AC (solid state) was used, which does not cause media obscuration. Because, according to some authors, charcoal particles adhere to dividing microspores or developing embryos, thus inhibiting their growth and development (Thomas, 2008).

Culture medium 1- standard prepare vitamins without AC

Culture medium 2- standard prepare vitamins +AC

Table 1. Composition of added vitamins

Standard vitamins according to Nich and Nich		B-complex of Doppelhertz	
Composition	Concentration mg/L	Composition	Content in 1 pill
Niacin (Vitamin B3)	5	Niacin (Vitamin B3)	21,6 mg NE
-		Pantothenic acid (Vit. B5)	18 mg
Pyridoxine (Vitamin B6)	0,5	Pyridoxine (Vitamin B6)	6 mg
-		Riboflavin (Vit. B2)	4,8 mg
Thiamin (Vit. B1)	0,5	Thiamin (Vit. B1)	4,2 mg
Folic acid (Vit. B9)	0,5	Folic acid (Vit. B9)	400 µg
Biotin (Vit. B7)	0,05	Biotin (Vit. B7)	150 µg
-		Cobalamin (Vit. B12)	3 µg

Culture medium 3 - B-complex in low concentrations without AC

Culture medium 4 - B-complex in low concentrations + AC

Culture medium 5 - B-complex in higher concentrations without AC

Culture medium 6 - B-complex in higher concentrations + AC

The experimental concentrations are consistent with the described concentrations of the included in their composition vitamins.

Methods

All tools, culture medium and mixtures of peat and perlite are autoclaved for 25 - 30 minutes at 1 atm. Syringe filters are used to sterilize the heat-sensitive components of the culture medium.

- Preparation of the explants - Flower buds of optimal length (equal sepals and petals) are selected and pre-treated at 4 °C for 24 to 72 h.

- Growing conditions - temperature 26-28°C in the light and 18-20°C in the dark; 16 hour dark/ 8 hour light.

Results - % regenerants according formula:

$K = X/Y \times 100\%$,

where X is the number of regenerants; Y number of flower buds

RESULTS AND DISCUSSION

The *in vitro* androgenesis is influenced by many factors, such as genotype, growing conditions, collection time, pre-treatments, induction and regeneration media and culture conditions. The combinations of these factors determine the efficiency of *in vitro* anther cultures (Bat et al., 2020; Lantos et al., 2022; Spasova-Apostolova & Masheva, 2020).

The study investigated the effect of different concentrations and composition of vitamins and AC on the initial stages of androgenesis and plant regenerants development.

The percentage of germinated plants in the two experimental years was different, in 2020 it was significantly lower. In the second year, 60 flower buds were cultivated and from 32 (more than 50%) regenerants were obtained.

This could be result on the climatic conditions during the two years of cultivation of the donor plants. Growth conditions of the donor plants play a significant role in determining the androgenic response different genotypes.

Plant species and varieties require different amounts of vitamins, while others require none at all. Thiamine, pyridoxine, nicotinic acid and myo-inositol are present in culture media and have no adverse effects on growth individually (Abrahamian & Kantharajah, 2011). Thiamine, for example, is essential for soybean, rice and tobacco crops, but not essential for peanut cells, which contain a high concentration of thiamine (Abrahamian & Kantharajah, 2011).

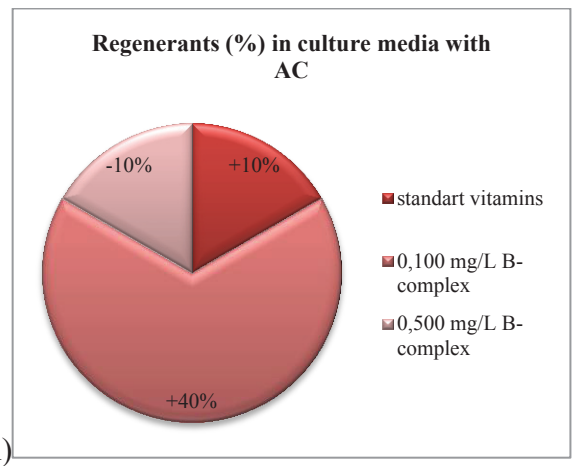
In the present study culture media 3, 4, 5 and 6, in addition to vitamins B3, B6, B1, B 9 and B7, also contains pantothenic acid (vitamin B5), riboflavin (vitamin B2) and cobalamin (vitamin B12). In the first year, germination of regenerants was found first in CM containing low and high concentration of B-complex, but the result was not confirmed in subsequent experiments, since in the second year

simultaneous germination was reported in all culture medium (Fig. 1).

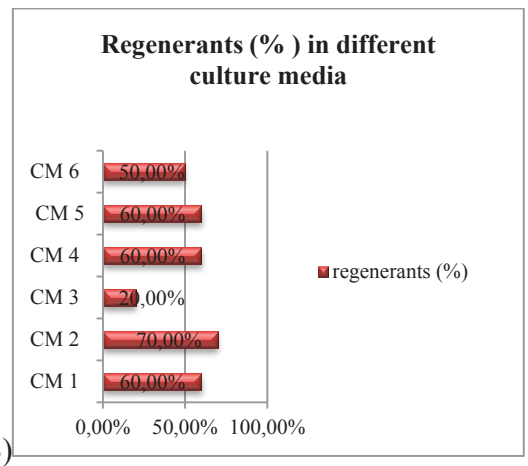
The results presented in (Fig. 1 B) showed the similar rate of regenerant (%) in CM 1, 2, 4 and 5 in the second year.

The biggest difference in the percentage of regenerants (40%) in CM with and without AC was found between CM 3 and 4 (with low concentrations of vitamin B-complex). In CM with higher concentration of vitamin B complex + AC the difference was lower 10%. In culture medium with standard vitamins + AC, 10% more regenerants are found (Fig. 1 A). The values of percentage distribution of regenerants in CM 1, 2, 4 and CM 5 confirms that B-complex vitamins could be used replacing the standard ones in the initial stages of androgenesis in the Krumovgrad ecotype.

Culture media with higher concentrations of B-complex vitamins had better developed regenerants than CM with lower B-complex concentration (Fig. 2).



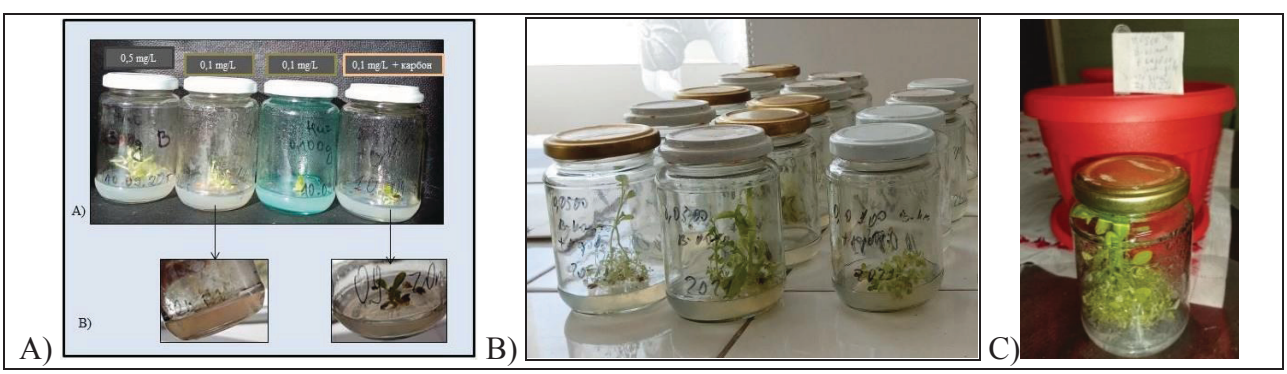
A)



B)

Flower buds	CM 1	CM 2	CM 3	CM 4	CM 5	CM6
	Standard vitamins without AC	Standard vitamins + AC	0, 100 mg/L in B-complex without AC	0,100 mg/L B-complex + AC	0,500 mg/L B - complex without AC	0,500-mg/L - B-complex + AC
Placed	10	10	10	10	10	10
Regenerants	6	7	2	6	6	5

Figure 1. Regenerants (%) in different culture media



A) B) C)

Figure 2. Regenerans CM of Nich and Nich

A number of studies have reported that addition of cobalamin to the culture medium favourably affects androgenesis by increasing haploid yield and improving embryo development (Jha et al., 2021). In the current studies, no negative effect of androgenesis was also found when using complex vitamins including cobalamin.

Riboflavin has various effects on plant rooting, both positive and negative. As more riboflavin is added rooting decreases in a linear fashion until it is completely inhibited (Abrahamian & Kantharajah, 2011).

In a study on the effect of vitamins (B2) on *in vitro* rooting of the almond peach hybrid clone GF 667 it was found that riboflavin did not stimulate adventitious rooting of the explants and rooting was much lower compared to controls. The regenerants showed tip chlorosis and necrosis at the highest concentrations of vitamin - B2 - 1.5 and 2.0 mg/L. Riboflavin acts on auxin photooxidation and inhibits rooting (Antonopoulou et al., 2005).

In the present study, a lower rooting rate was found at higher concentrations of B -complex. The root system develops most strongly in CM 4 with 0,100 mg/L B-complex and AC (Fig. 3 B), followed by CM 6 with 0,500 mg/L B-complex and AC. Riboflavin (B2) is related to the formation of a more developed root system, at lower concentrations and probably the AC manages to adsorb a part, thus providing them.

Riboflavin has been found to inhibit callus formation, but can improve shoot growth and quality (George et al., 2007). In the present study, callus formation was found only in 1 sample at the low concentrations of B complex with AC. The results for regenerants (%) in all CM showed that vitamins B5, B2 and B12 can be applied and have no negative effect, but are not essential.

Regenerants from CM 5 and 6 with higher concentrations of B-complex were the first transplanted in pots for adaptation in 2022. They had a very well developed vegetative and root system (Fig. 2 C).-

Transcriptase analyses in wheat have demonstrated that AC can significantly increase the molecular mechanisms underlying growth. Suppression of growth inhibitory gene expression by regulating plant hormone signalling (Dong et al., 2020).

In CM with low concentrations of B-complex, regenerants are better developed when AC is added. No visual difference in the development of plants in CM at higher concentrations of B-complex with and without AC. Probably, the combination of low concentrations of vitamins with AC has a favorable effect on the initial stages of the development of androgenesis in plants of the Krumovgrad ecotype.

The results of the present study confirm that a culture medium with AC favourably affects the initial stages of the development of androgenesis in plants of the Krumovgrad ecotype, especially at lower concentrations of vitamins.

In this experiment, the largest number of regenerants with a highly developed root system were reported in CMs with B - complex vitamins and active charcoal.

Figure 3. Root system in different CM

CONCLUSIONS

B-complex vitamins could be used instead of standard vitamins in the initial stages of androgenesis of Krumovgrad ecotype tobacco.

The use of higher concentrations of B-complex stimulates faster development of regenerants.

In culture medium with lower B-complex concentrations, regenerants are better developed when of activated charcoal is added.

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