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The effects of antibiotics and antifungals added to plant culture media of *Triticosecale wittmarck*

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SUMMARY. The experiment aimed to study the antibiotics or antifungals effect to prevent the infection in *Triticosecale wittmarck* micropropagation, without affecting the inoculum. After identifying the pathogens, which are frequently affecting the *in vitro* culture media, respectively *Clostridium* sp. and *Cladosporium* sp., we made the antibiogram and antifungal susceptibility testing. Among antibiotics and antifungals, to which one of them were found to be sensitive, we chose to add the following to the culture media: V_1-gentamicin (GEN) - 40 mg/L, V_2-tetracycline (TET) - 10 mg/L, V_3- ciprofloxacin (CIP) - 40 mg/L, V_4-clotrimazole (CLO) - 50 mg/L, V_5-fluconazole (FLU) - 50 mg/L, V_6-terbinafine (TER) - 25 mg/L, having the culture medium, without anti-contaminants additions, as control lot. At the end of the experiment we concluded that anti-contaminants, used in concentrations recommended by literature, have prevented infections, but reduced the capacity for germination rate of triticale. Just terbinafine can be added in triticale culture media, but it is necessary to study other lower concentration that does not affect the growing of inoculum.

Keywords: antibiotics, antifungals, culture media, *Triticosecale*

Introduction

In vitro cultures, of all kinds, are exposed to contamination risks with different kinds of microorganisms. In agar media, the chemical composition and quality are less affected, respectively fungi mycelia or bacteria invasion are slower than compared to liquid media. However, usually in about one week after inoculation, the hyphae fungi invade the phyto-inoculum, the molds dampening the vitrocultures they infected (Turcuș and Cachiță-Cosma, 2009). Usually, the cultures initiated from explants taken from plant material grown in septic conditions, show a high occurrence of accidental infestations (Cachiță-Cosma and Ardelean, 2009).

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The reason of occurrence in such infections may be the exogenous (deficiencies in working technique) or endogenous nature (germs spread deep down in the intern structure of the explant, bacteria or mycosis located intercellular or endocellular) (Cachiță-Cosma and Ardelean, 2004). The micopropagation success will depend not only on preventing primary infections, but also preventing the occurrence of secondary infections, possible a few weeks after the inoculation.

Protecting the plants against these agents is mainly made with antibiotics and antifungals (Makovitzki *et al.,* 2007). For eliminating the infections, it is used antibiotics and antifungals treatment, or cultivating them on culture media with additional anti-contaminants (Thomas, 2004; Cachiță-Cosma and Ardelean, 2009; Kulkarni and Krishnamurthy, 2009; Smith, 2013), as mixture of methylchloro-isothiazolinone, methylisothiazolinone, magnesium chloride and magnesium nitrate (Guri and Kishor, 1998). The first research with antibiotics added in culture media involved vanilin and its derivatives (Knudson, 1974; McAlpine, 1974 cited by Thurston *et al.,* 1979).

Throughout the years, in many researches were made efforts for introducing certain antibiotics or antifungals, in order to prevent contamination of plants *in vitro* cultures (Arditti, 2008). Concentrations used by researchers varied: 0.1 mg/L metalaxyl, 1.0 mg/L actinomycin D, 2.5 mg/L amphotericyn B, 5 mg/L sodium omadine, 100 mg/L paromocyn sulfat, penicilin G, streptomicyn sulfate or pentochloronitrobezene. There are anticontaminats wich can be used in large concentrations: 300 mg/L ticarcilin, 400 mg/L ampicilin, 500 mg/L carbenicilin, 800 mg/L geneticin, 1000 mg/L cefotaxime, but most recommended concentration is between 10 –50 mg/L: tetracycline hydrochloride, thiabendazole, benomyl, cycloheximide, aureomycin, bavistin miconazole, mytomicin C, neomycin sulfate, rifampicin, vancomicyn (Arditti, 2008). Adding 50 mg/L gentamicin to the medium seemed to be useful, but not for the latent bacteria (Thomas, 2004). Amphotericyn B (92.7% pure,10 ppm), nystatin (4020 units mg/l, 25 ppm) and sodium omadine (90% pure, 5 ppm), penicilin G (1595 unit mg/L, 100 ppm), benomyl (50 % pure, 50 ppm), dowicide (97% pure, 5 ppm), gentamicyn (50 ppm), vancomicyn (50 ppm) used individually delayed *Cymbidium* shoots (Brown *et al.*, 1982).

The purpose of the present research is the study the antibiotic and antifungal effect on preventing infection of *in vitro* cultures, without affecting the development of *Triticosecale wittmarck* inoculum.

Materials and methods

Since the infection management techniques efficiency begins with a proper identification of the disease and/or the causal organism (Leonberger *et al.*, 2016), we have started to identify the pathogens in the *in vitro* cultures and the anti-contaminants to which they are sensitive (Cosma and Petruș-Vancea, 2017), to add them later in the culture media. Thus, two different species of microorganisms,

namely *Clostridium* genus and a fungus of *Cladosporium* genus, have been identified by diffusometric sensitivity testing methods (Costache and Colosi, 2008; Cercenado and Saavedra- Lozano, 2009). In the previous study (Cosma and Petruș-Vancea, 2017) we found out that the bacteria was sensitive to ciprofloxacin, levofloxacin, gentamicin, vancomycin, tetracycline, chloramphenicol, trimethoprim - sulfametoxazole, imipenem, and the fungus was sensitive to terbinafine, fluconazole, clotrimazole and posoconazole.

Plant material used in our experiments consists in triticale *(Triticosecale wittmack)* caryopses and research design is presented in table 1.

Culture media was standard Murashige-Skoog (1962) with Gamborg (1968) vitamins, solidified with agar-agar 8 mg/L and 5.7 pH, without growth regulators, which was placed in 5 mL glass containers with a size of 2/7 cm.

Antibiotic and antifungal concentrations were determined following references in the literature (Arditti, 2008). Understanding the effects of many important antibiotics in the plant physiological activity is still limited (Opriș *et al.,* 2013*).* In the orchid culture media, Thurston *et al.* (1979) recommended the combined introduction of anticontaminants, as well: benlate + nystatin + penicilin G + gentamycin + sodium omadine + graphite and the development of stock solutions for an easy work.

Table 1.

Experimental protocol

Experimental variants	Disinfection	Growth conditions	Measurement periods	Measurement types
V_0- without anti-contaminants (*control*) V_1-GEN-40 mg/L V_2-TET-10 mg/L V_3-CIP-40 mg/L V_4-CLO-50 mg/L V_5-FLU-50 mg/L V_6-TER-25 mg/L	15 min. 2% sodium hypochlorite +Tween 20 submersion, then followed by 5 sterile water washes (25 min.)	- 22-24 ˚C - white fluorescent lighting, light intensity 91800 μmol m^{-2} s^{-1} 16 h photoperiod	3 days 7 days	Embryonic root L. Coleoptile L. Leaf L. Germination % Survival % Infection level

Note: GEN-gentamicin, TET-tetracycline, CIP-ciprofloxacin, CLO-clotrimazole, FLU-fluconazole, TER-terbinafine, L.- length.

The inoculation was carried out in the sterile room, using horizontal flow cabinet, following the asepsis rules (Petruș-Vancea *et al.,* 2013).

Biometric data were mathematically processed in the Microsoft Excel 2013. The *t* test was performed using the SPSS for Windows to identify the significance of the difference from the control lot ($p<0.5$).

Results and discussion

At 3 days after germination, the germination percentage was increased in the control lot (V_0) and in the presence of gentamicin (V_1). The lowest germination rate was recorded at the *in vitro* group placed on culture media supplemented with tetracycline (V_3) (Fig. 1). The effect of tetracycline was reported to be inhibitory on germination by Turdeanu and Petruș-Vancea (2015), affecting *in vivo* triticale germination, but also by Mocanu and Petruș-Vancea (2016) in *in vitro* cultures.

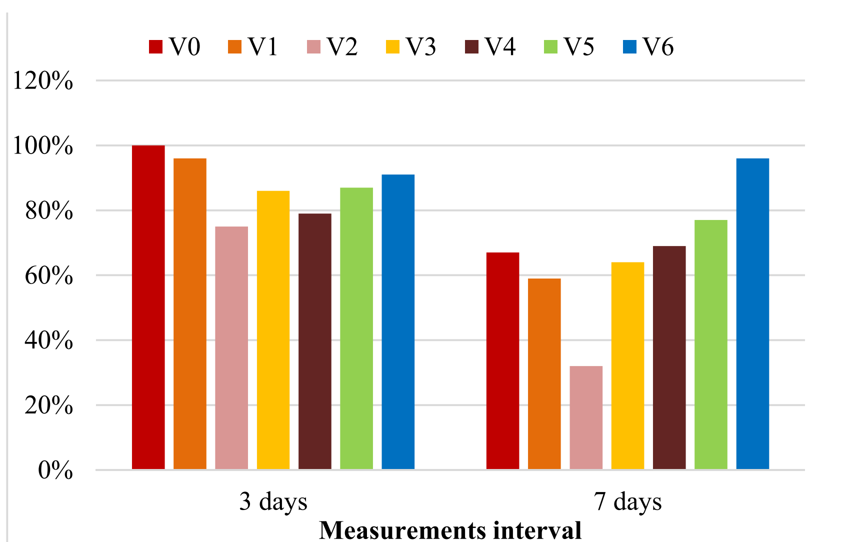

Figure 1. Percent expression of the *Triticosecale wittmarck* ***germination*** at **3 days** and the ***survival*** at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine, compared to the total of inoculated containers, which represents 100%.

Although germination process satisfactorily started, at 7 days after germination, there were large losses due to germinated plants necrosis, so the survival rates were diminished in all experimental variants, including the control, with the exception of the plants lot placed on culture media with the addition of terbinafine, a fungicide that caused necrosis to *Sequoia* minicuttings (personal data).

A different reaction was identified according to the ontogenetic plant period, respectively in the first days of life, immediately after germination (3 days) and during the growth period (7 days), when greater inhibitions could be measured (Fig. 1).

Even at 3 days after inoculation, *Cladosporium* infections occurred, especially on the control and antibiotic added to media, but also on the Clotrimazole antifungals addition. Infections in this case of culture initiation are caused by the cariopse explants insufficient sterilization, which conduct to over infection, to the impossibility of the fungicide to cope with the infection.

However, there was only one experimental variant, V_6, namely the terbinafine addition, with any infection (Fig. 2).

Moreover, the highest survival rate was recorded on the terbinafine medium, at 7 days after inoculation (Fig. 1), even higher than to the control. Instead, the growth indexes were small compared to the control (Table 2 and Fig. 3).

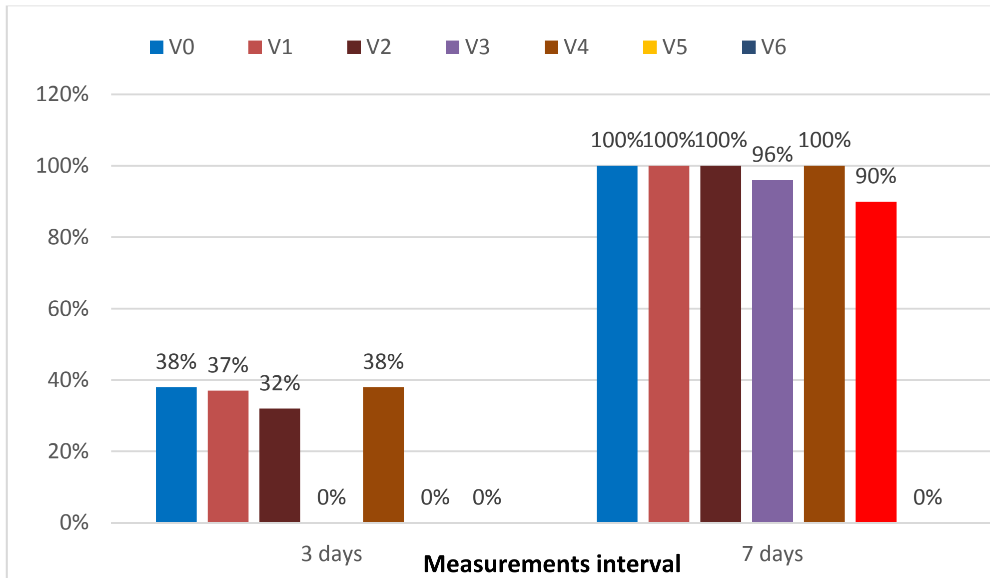

Figure 2. Percent expression of the *Triticosecale wittmarck* ***infection*** at **3 days** and **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine, compared to the total of inoculated containers, which represents 100%.

Although the highest survival values were recorded in the terbinafine lot (V_6), respectively the null infection rate, from the plant growth indexes point of view, the highest inhibitions were registered (Table 2 and Fig. 3). Infected plants continued the *in vitro* growth process.

Cefotaxime was shown to eliminate contamination with *Xanthomonas campestris* pv.*pelargonii* and stimulate the growth of the *Pelargonium x domesticum* cv. 'Grand Slam' plant tissue cultures (Barrett and Cassells, 1994).

Table 2.

Plant growth indexes values, at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine (L.- length)

Types	Average (cm) ± standard deviation			Difference from control (cm) / significance of difference (p)					
	Root L.	Coleoptile L.	Leaf L.	Root L.		Coleoptile L.		Leaf L.	
V0	1.96±0.60	2.46±0.42	1.48±0.92		**p**		**p**		**p**
V1	1.93±0.72	2.78±0.43	1.23±0.91	-0.03	Ns	0.32	Ns	-0.25	Ns
V2	1.52±0.63	1.98±0.79	0.78±0.75	-0.44	Ns	-0.48	Ns	-0.70	Ns
V3	0.99±0.59	2.03±0.83	0.17±0.35	-0.97	*	-0.43	Ns	-1.31	***
V4	1.10±0.37	2.50±0.64	0.27±0.56	-0.86	Ns	0.04	Ns	-1.21	**
V5	1.50±0.58	1.35±0.55	0.58±0.61	-0.46	Ns	-1.11	*	-0.90	*
V6	0.4±0.18	0.7±0.26	0.00	-1.56	***	-1.76	***	-1.48	***

Note: $\bar{x} \pm S_{\bar{x}}$ - average (cm) ± standard deviation); p (significance): p>0.5 - non significance (Ns), p<0.5 - significance (*), p<0.1 - distinct significance (**), p<0.01 – very significance (***).

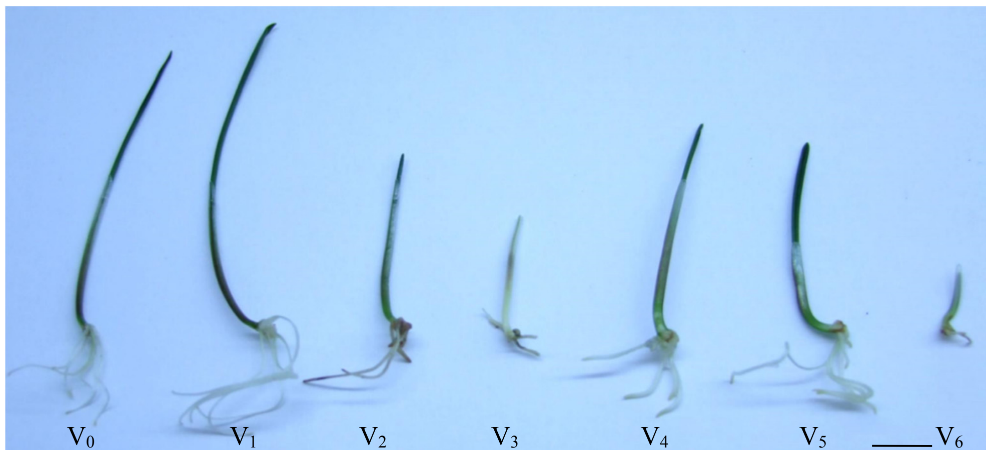

Figure 3. *Triticosecale wittmarck in vitro* cultivated plants at **7 days** after inoculation on the following culture media: V_0 – control, MS-G, without anti-contaminants; V_1 – MS-G + gentamicin; V_2- MS-G + tetracycline; V_3- MS-G + ciprofloxacin; V_4- MS- G + clotrimazole; V_5- MS-G + fluconazole; V_6- MS-G + terbinafine (bar means 1 cm).

Conclusions

1. Primary *in vitro* cultures initiated from triticale caryopses (*Triticosecale wittmarck*) in culture media with added antibiotics namely: gentamicin 40 mg/l, tetracycline 10 mg/l, ciprofloxacin 40 mg/l or antifungals like clotrimazole 50 mg/l and fluconazole 50 mg/l showed fungal infections.
2. Terbinafine 25 mg/l completely prevented the infections, but generated significant inhibitions of the growth indexes, therefore we consider necessary for the future to study the effect of a smaller concentration, which would not affect the plantlets growth.
3. It is necessary to continue researches in order to determine the antibiotics and antifungal combination, in a suitable concentration for preventing infections and proper growth of the *in vitro* plantlets, according to their species.

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