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The infuence of Imidazole fungicides on multiplication *in vitro* of pyrodwarf pear rootstock

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Abstract

Pyrodwarf, low vigorous pear rootstock was used as a model plant in these investigations. Under the *in vitro* conditions, successful micropropagation of this genotype is largely based on the nodal transplantation of shoots due to low potential of lateral shoots to form. Therefore, the objective of these investigations was to study the influence of imidazole fungicide Prochloraz on the multiplication. The commercial chemical SPORTAK 45–E–450, which contains active substance Prochloraz (N-propyl-N-[2-(2,4,6-trichlorphenoxy)-ethyl]imidazole-1-carboxamide), was used as the source of the imidazole fungicide. The experiment was performed during the multiplication phase, and it involved the study of 19 types of media which included MS macro and micro salts, different prochloraz concentrations, BAP, IBA and GA_3. Prochloraz involved 3 concentrations (1, 5 and 10 µM), either individually or combined with BAP (4.4 µM), IBA (5 µM) and GA_3 (0.3 µM). Upon the second subcultures, the parameters of the shoot multiplication, multiplication index and the length and the number of leaves on axial and lateral shoots were determined. Fresh and dry shoot weight, i.e. callus, stem and leaves were also checked. The highest multiplication index (1:2.72) was obtained at the medium which contained 10 µM of prochloraz combined with 4.4 µM BAP and 0.3 µM GA_3, individual multiplication index being even up to 1:6. Individual or IBA combined application of fungicides affected the shoot rooting of the Pyrodwarf (up to 100%). The obtained results suggest that Prochloraz intensifies the effect of the added exogenous BAP on *in vitro* multiplication of the Pyrodwarf pear rootstock and could be recommended for micropropagation of this rootstock.

Keywords: imidazole fungicides; *in vitro;* multiplication; pear rootstock; rooting

Abbreviations: MS – Murashige and Skoog (1962) medium; PRO – prochloraz; BA – 6-benzyladenine; IBA – indole-3-butyric acid; GA_3 – gibberellic acid, FW – Fresh weight; DW – Dry weight of explants; Hormone free – HF

Introduction

The application of fungicides in *in vitro* culture has shown that some of these substances may exhibit organogenic and morphogenic effects on *in vitro* plants. Thus, it has been discovered that the imidazole fungicides, such as imazalil (IMA), prochloraz (PRO), triflumizole (TRI), and triazole retardant paclobutrazol (PBZ) either intensify the effect of exogenous cytokinins or inhibit biosynthesis of the gibberellic acid. A number of interesting side effects of the imidazole fungicide IMA can be observed in diverse tissue cultured plant species, i.e excessive shoot formation in *Araceae*, inhibition of bushiness in *Gerbera*, restoration of normal embryo development in

Table 1. Media used in the experiment

Nº of medium	**BAP µM**	**Prochloraz µM**	**GA_3 µM**	**IBA µM**
1	0	0	0	0
2	0	0	0.3	0
3	0	5	0	0
4	0	5	0.3	0
5	0	10	0	0
6	0	10	0.3	0
7	4.4	0	0	0
8	4.4	0	0.3	0
9	4.4	5	0	0
10	4.4	5	0.3	0
11	4.4	10	0	0
12	4.4	10	0.3	0
13	0	1	0	0
14	0	1	0.3	0
15	4.4	1	0	0
16	4.4	1	0.3	0
17	0	1	0	5
18	0	5	0	5
19	0	10	0	5

Citrus and histogenic instability of *Ficus benjamina* L. (*Moraceae*) chimeras (Werbrouck et al., 2001).

On testing fungicides aiming at avoiding fungal contaminations in tissue culture, Werbrouck and Debergh (1996) observed a cytokinin-like effect of the imidazole fungicides, IMA and PRO, on *Spathiphyllum* and *Anthurium*. The major symptom was excessive shoot formation at the base of the plant. On cytokinin-free medium buds were not induced with IMA, but it was later observed that IMA also enhanced the effect of other cytokinins as zeatin and even thidiazuron in *Spathiphyllum floribundum* (*Araceae*). PRO had the same effect. PRO and BA interacted similarly in *Anthurium andreanum,* also repesentative of the *Aracae* (Werbrouck and Debergh, 1996).

Besides varying hormonal composition of the medium, Pyrodwarf pear rootstock displayed very low multiplication index under the *in vitro* conditions (Ružić et al., 2004). Aiming at the improvement and advancement of the multiplication *in vitro* and bearing in mind the activity of imidazol fungicides, we orientated us towards discovering their influence on multiplication of this popular low vigorous rootstock. At the same time it will be the first results obtained with this pear rootstock and imidazol fungicides.

Materials and Methods

Plant material

Very popular low vigorous pear rootstock Pyrodwarf (clone BU 5-18) the breeder of which is Jacob B Helmut (singled out from the cross of Old Home x Bonne Louise d'Avranches, genus: *Pyrus*, family: *Rosaceae*, in Geisenheim Research Institute (Geisenheim, Nemačka) was used as the model plant for the purpose of this study. The patented name of this rootstock is Rhenus 1 (Jacob, 2002). In order to avoid the effect of residues from the previous medium, Pyrodwarf shoots were subcultured prior to placing on specific media, and morphometric measurements were taken from the second subculture.

Media

Upon the multiplication of sufficient number of shoots (Ružić et al., 2004) they were placed on the media MS containing imidazole fungicide PRO (Table 1). The commercial chemical SPORTAK 45 –E–450, which contains active substance N-propyl-N-[2-(2,4,6-trichlorphenoxy)-ethyl] imidazole-1-carboxamide – Prochloraz (molecular formula $C_{15}H_{16}C_{l3}N_3O_2$), was used as the source of the

Table 2. Multiplication parameters of Pyrodwarf shoots

Nº of medium	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)	Nº of leaves of axial shoot	Nº of leaves of lateral shoots
1	1.00 d*	1.31 fg	-	13.22 defg	-
2	1.00 d	1.28 g	-	12.06 efg	-
3	1.00 d	1.48 efg	-	10.78 g	-
4	1.00 d	1.41 fg	-	11.67 fg	-
5	1.00 d	1.34 fg	-	11.89 fg	-
6	1.00 d	1.33 fg	-	11.11 g	-
7	1.22 d	2.53 ab	1.00 b	16.33 abc	5.75 b
8	1.33 cd	1.97 cd	0.70 b	14.94 bcde	5.50 b
9	2.28 a	2.84 a	1.64 a	18.83 a	8.18 a
10	1.72 bc	2.36 b	1.12 b	17.78 ab	7.69 a
11	2.61 a	2.24 bc	0.98 b	17.11 abc	6.57 ab
12	2.72 a	2.29 bc	1.64 a	19.06 a	8.29 a
13	1.00 d	1.63 defg	-	14.39 cdef	-
14	1.00 d	1.66 def	-	16.11 abc	-
15	1.83 b	1.78 de	1.03 b	15.42 bcd	6.93 ab
16	1.50 bcd	1.84 de	0.99 b	16.17 abc	7.89 a
17	1.00 d	0.74 h	-	12.06 efg	-
18	1.00 d	0.79 h	-	12.28 efg	-
19	1.00 d	0.74 h	-	13.06 defg	-

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's Multiple Range Test.

imidazole fungicide. As SPORTAK 45–E–450 contains 450 g of PRO the calculations were related to this specific quantity. All the media contained agar at concentration of 7 g l^{-1} and sucrose 20 g l^{-1}. Prior to autoclaving, the pH value of all media was adjusted to 5.75 with 0.1 N KOH. The media were sterilized in an autoclave for 20 min at 120^{o}C. PRO was added through filter-sterilization by Millipore filter of 0.22 µm upon media autoclaving.

Multiplication parameters

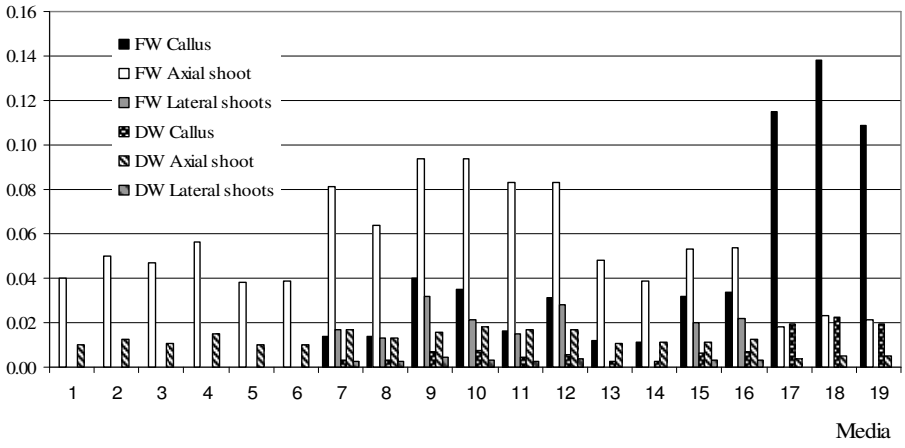
Multiplication parameters were determined by standard morphometry. Shoots smaller than 0.5 cm were not taken into consideration. The following multiplication parameters were monitored: multiplication index, length of axial and lateral shoots, number of leaves on axial and lateral shoots. Some specific issues, such as colour of leaves and callus size, the incidence of chlorosis or necrosis along with the occurence of rhyzogenesis, i.e. roots, uncommon for this phase of micropropagation, were also monitored.

Fresh weight and dry weight of explants

Fresh and dry shoot weight (callus, stem and leaves) was also determined. Upon the removal from the medium, shoots were rinsed in distilled water, dried with filter paper and their FW was checked. As for the DW, shoots were dried in an oven at 65-70^{o}C for 48 h.

Cultural conditions

The cultures were grown under a 16 h photoperiod, with a light intensity of 41 mol $m^{-2}s^{-1}$ on the culture surface provided by cool white fluorescent tubes 40 W, 6.500^{o}K in strength, at temperature 25 ± 1^{o}C. Ten culture vessels x 5 uniform shoots x 2 replications were used for each tretment (19 treatments - the total of 1,900 shoots). The data were analysed by ANOVA as well as by the individual Duncan's Multiple Range Test.



Graph 1. Fresh and dry shoot weight of rootstock pyrodwarf (in g)

Results

In this trial, the highest multiplication index was obtained on the medium 12 with BA 4.4 µM, PRO 10 µM and GA_3 0.3 µM, 1: 2.72, which is for 1.6 times higher than the most favourable multiplication of this genotype obtained by varying of the hormonal composition (Ružić et al., 2004). The length of the axial shoot was highest on the medium containing both 4.4 µM BA and PRO 5 µM but not GA_3. Multiplication index was also good on this medium (Table 2).

Plants grown on these media were large, with elongated axial shoots and well developed deep green leaves (Fig. 1 a). The calluses were light creamy in colour, firm, nodular. Individual multiplication index was 1:6, particularly on medium 12. Applied individually or combined with GA_3 and without BA, PRO did not affect the multiplication (Table 2).

Fresh and dry callus weight was highest on the medium containing only PRO 5 µM and IBA 5 µM (Graph 1). However, FW and DW of axial shoot, lateral shoots and leaves was highest on the medium with 4.4 µM BA and PRO 5 µM, which also recorded the highest length of the shoots.

On the medium containing PRO 1 µM and IBA 5 µM rhysogenesis occurred and 100% of plants were rooted. The occurrence of rhyzogenesis was observed on the other media as well. These media did not contain BA, the rooting rate ranging from 11.11% (media 4 and 6) to 100% (media 17 and 19). Thus, the highest root formation was observed on the medium with 1, 5 and 10 µM PRO combined with IBA (Tab. 3). Short stem, large and wide leaves of deep green colour are common characteristic of all plants rooted on these media.

The callus was large, white, soft and spongy. The roots were short, thick, white, radially spread, without secondary roots (Fig. 1 b).

On the media containing only PRO 1 µM, where rhyzogenesis was also observed, plants were large, well developed, with long stems and wide deep green leaves. The callus was small, hardly visible, nodular, firm, yellow in colour. The shoot base is almost encircled with the callus, the roots being long, white, without the occurrence of the secondary roots, emerging from the shoot base (Fig. 1 c). Similar, very long roots are also formed on HF media and on those containg only GA_3 (Tab. 3; Fig. 1. d).

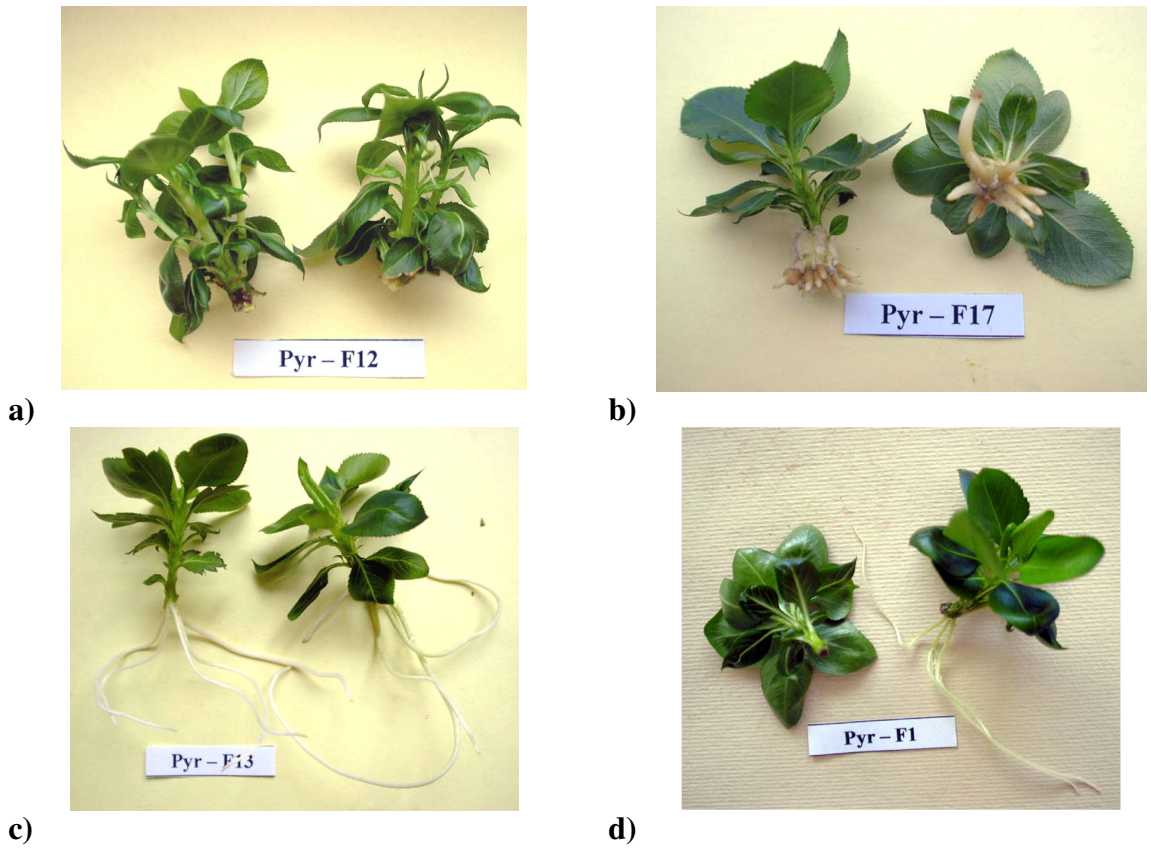
Discussion

Imidazole fungicides induce morphogenetic and organogenetic responses, characterised by abundant adventitious bud formation. The first working hypothesis was that a cytokinin-like effect was involved (Werbrouck et al., 1999). In our experiment, however, it was only for the induction of the shoot formation of Pyrodwarf rootstock in the presence of the cytokinin BA that PRO was really effective. Another hypothesis claimed that the gibberellins might act as a „brake“ on the shoot inducing potential of cytokinins, a brake which is released by adding GA-inhibitors, such as IMA or PBZ (Werbrouck et al., 1999). The imidazol fungicides IMA, PRO and TRI strongly enhanced the shoot inducing effect of BAP in *Spathiphyllum floribundum* (Werbrouck et al., 1999). Neither these fungicides nor PBZ showed cytokinin effects on cytokinin – free medium and this suggests an

Table 3. Parameters of the rooted pyrodwarf shoots

No° of media	Rooting rate (%)	Average number of roots per rooted plant	Average length of roots per rooted plant (cm)	Average FW of roots per rooted plant (mg)	Average DW of roots per rooted plant (mg)
1	16.67 cd*	2.33 cd	4.56 a	28.7 b	2.6 b
2	16.66 cd	1.67 d	4.92 a	42.6 ab	3.8 ab
3	66.67 b	2.42 cd	2.62 b	26.2 b	2.9 b
4	11.11 d	3.00 cd	3.63 ab	45.6 ab	4.4 ab
5	27.78 c	4.40 c	0.46 c	18.0 b	2.0 b
6	11.11 d	1.50 d	4.23 a	43.2 ab	3.8 ab
13	72.22 ab	3.30 cd	4.57 a	72.3 a	6.7 a
17	100 a	9.39 a	0.53 c	52.0 ab	7.0 a
18	94.44 a	8.08 ab	0.46 c	38.3 b	5.5 ab
19	100 a	6.83 b	0.40 c	27.6 b	4.4 ab

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's Multiple Range Test.



a) b) c) d)

Fig 1. Shoots of Pyrodwarf rootstock on medium with: (a) BA 4.4 µM; PRO 10 µM; GA_3 0.3 µM (b) PRO 1 µM; IBA 5 µM (c) PRO 1 µM (d) HF

interaction between imidazoles and applied cytokinins (Werbrouck and Debergh, 1996). It is well known that gibberellic acid inhibits the normal induction of new shoots by BA and this phenomenon has often been reported for other crops and is reviewed by George (1993).

Werbrouck et al. (1996) also interpreted that the endogenous gibberellins block the full expression of the shoot-inducing potential od exogenous cytokinins like BA. Adding IMA, PRO or PBZ removes the block by inhibiting GA biosynthesis and as a consequence shoot proliferation is enhanced. The absence of shoots when IMA or PRO are added to a BA-free medium indicates that the level of endogenous cytokinins is not adequate to induce shoots, even when the blocking effect of endogenous GA is alleviated (Werbrouck et al., 1996). Applied either combined with IBA or individually, PRO influences the formation of large callus mass as well as very high rooting rate of the Pyrodwarf rootstock, particularly when combined with IBA. The rooting was accompanied by specific characteristics of the formed roots - from short and thick radially spread roots (on media containing combined PRO and IBA) to long and thin roots (on media containing only PRO). However, Werbrouck et al. (1999) observed that root elongation was strongly inhibited by IMA and the use of an imidazole fungicide resulted in the development of thicker roots. According to Ružić et al. (2004), grown on the rooting media with IBA and GA_3, Pyrodwarf rootstock resulted in 92% of the rooted plants, the roots of which were short and thick, which excludes the influence of PRO.

Conclusion

The effect of imidazole fungicide PRO *in vitro* on shoot multiplication of Pyrodwarf rootstock can only be observed in the presence of exogenous cytokinins such as BA. It is most likely that imidazole fungicides, such as PRO, inhibit the biosynthesis of gibberellins and cytokinins, consequently manifesting their full shoot/induction potential. PRO applied either individually or combined with IBA affects the root induction *in vitro*. The application of imidazole fungicides for the *in vitro* optimization of micropropagation of some genotypes may result in the invention of new chemicals which may be practical for the purposes of tissue culture. Investigations of this kind add up greatly to the general knowledge on how growth substances act.

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