The effect of growth regulators on the rooting of shoots of the peach rootstock Ishtara in *in vitro* conditions

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ABSTRACT: The effect of indolyl-3-butyric acid (IBA) and paclobutrazol (PP 333) on the rooting of shoots of the Ishtara peach rootstock was examined in *in vitro* conditions in light. In the first stage of the experiments the effect of the IBA concentration (0, 0.75, 1.5 mg/l) was studied and in the second stage the effect of the interaction of IBA (0.75 mg/l) and PP 333 (0.06 and 0.12 mg/l) in the MS medium, which is optimal for the rooting of the peach rootstock Ishtara. During shoot cultivation we evaluated root formation (average length and number). During rhizogenesis the production of ethylene, ethane and CO_2 by the shoots and the level of abscisic acid (ABA) in the shoot base was monitored in *in vitro* conditions. Shoots cultivated on the MS medium with 1.5 mg/l of IBA produced the highest number of roots and the longest roots that produced the lowest level of ethylene and CO_2 and the level of ABA was the lowest in the bases.

Keywords: IBA; PP 333; ethylene; ABA; *Prunus persica* L.; rooting *in vitro*

The cloning of fruit tree species *in vitro* is a process that is introduced all over the world (FARI et al. 1996) with the aim to obtain virus-free and identical plant material. The selection of peach rootstocks (*Prunus persica, P. domestica, P. armeniaca, P. amygdalus* and their crosses) is based on the soil properties, calcium content in the soil and the incidence of nematodes (*Meloidogyne arenaria, M. incognita, M. javanica*). The Ishtara rootstock tolerates humid soil and is nematode-resistant, the reason why the propagation of this rootstock by means of the explant technique in conditions *in vitro* is very promising. As Ishtara is a rootstock with a high propagation capacity, but different rooting (NÉMETH 1981; JONES, HOOPGOOD 1979; DRUART 1980; KARHU, ULVINEN 1995), the process of rhizogenesis was studied under *in vitro* conditions.

In 1912, on the basis of his morphological experiments, DOSTÁL reported that specific regulating effects arising from the growing axillary buds (the effect of auxins) boost the formation of adventitious roots, and in 1934 THIMANN and WENT announced that lexogenously applied IBA stimulated the formation of adventitious roots.

Many authors who studied the correlation between rhizogenesis and ethylene frequently achieved different results. As early as in 1933 ZIMMERMANN and HITCHCOCK proved the stimulating effect of ethylene on the formation of adventitious roots; RADIN and LOOMIS (1969) discovered the inhibiting effect of ethylene (2 to 3 µl/l) on the formation of lateral roots in radish; and DIMASI-THERIOU et al. (1993) reported that a concentration of 0.01–10 µl/l of ethylene increased the number of shoots and adventitious roots in petunia leaf regeneration.

A frequent problem of *in vitro* cultivation in pots is not only the high concentration of ethylene and ethane released by damaged cells that is considered as the indicator of lipid peroxidase (CELIKEL, VANDOORN 1995) but also the CO_2 concentration. BALLA et al. (2003) examined the process of rooting influenced either by an addition of saccharose into the medium or application of CO_2 into the cultivation area (3–4%); they discovered that an increased level of atmospheric CO_2 in parallel with a reduced level of saccharose in the medium resulted in the same % of rooting of cuttings of apple and prunus as if the medium contained 60 g/l of saccharose.

According to GRANT et al. (1992) a higher content of CO_2 stimulated the closing of stomata, after which transpiration in the plants decreased, increasing the water potential of the cuttings; at the same time the starch level in the plants increased, apparently due to a higher partial pressure created by the applied CO_2. Although the increased starch concentration increased the dry matter content in *Correa schlechtendalii* (Behr) by 70–90%, in *Chamelaucia* this increase was only 10–30% and that is why the authors incline to believe that increased rhizogenesis is due to the improved water conditions in the plant.

It is not clear yet whether abscisic acid generated in the roots and with consequent transport through the xylem and regulation of leaf growth, plays the primary regulating role in the formation of adventitious roots

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(DAVIES et al. 1994) or, on the contrary, whether abscisic acid is produced by the leaves and transported through the phloem into roots whose growth and development it influences (HOAD 1975). It is well known that a high level of ABA inhibits leaf growth (ALVES, SETTER 2000) and stimulates elongation root growth at low water potentials and low ethylene production (SPOLLEN et al. 2000). LA MOTTE et al. (2002) reported that the relation between the level of abscisic acid and indolyl-3-acetic acid in the leaves of *Coleus blumei* decreased during leaf growth in proportion with the correlation coefficient 0.91.

In the late 1970s information appeared about a preparation indicated as PP 333, the active ingredient of which is N-dimethylaminosuccinamic acid, a compound of triazole, paclobutrazol (ENGLAND 1978), bearing the commercial name Cultar; it inhibits gibberellin biosynthesis, increases stress tolerance and slows down senescence (DAVIS et al. 1988). Paclobutrazol can therefore be used in horticulture as a morphoregulator (HRADILÍK, FIŠEROVÁ 1986, 1987). The treatment of stem cuttings with PP 333 had a positive effect on rhizogenesis (DAVIS et al. 1988; KRÁLÍK, ŠEBÁNEK 1989; ŠEBÁNEK et al. 1991).

On this information we based our studies of the effect of exogenous IBA and paclobutrazol on the level of abscisic acid in the base of the Ishtara cutting and the production of ethylene, ethane and carbon dioxide in *in vitro* conditions during rhizogenesis in light.

MATERIAL AND METHODS

For the experiments we used the polyvalent peach rootstock Ishtara – ([*P. cerasifera* × *P. japonica*] × [*P. cerasifera* × *P. persica* F1-322 × 871]), which has upright stems and hardly any growth recovery – the so-called stem-forming variety. This variety is resistant to nematodes on humid soil. The growth of peach trees on this rootstock is 20% weaker than on peach seedlings, they enter the fruit-bearing stage more quickly and have a positive effect on the size of the peach fruit. Propagation from woody and semi-woody cuttings is very good. It is more resistant to asphyxia than the peach tree, yet it is more suitable for cultivation on lighter soil. Older trees are more sensitive to an increased Ca content in the soil (VACHŮN 1996).

The Ishtara rootstock explants were derived from the broken buds of annual shoots (4 to 5 nodal sections) on a modified DKW medium (DRIVER, KUNIYUKI 1984; ALSALIHY et al. 2003a,b) – agar 7 g/l, saccharose 30 g/l, $FeSO_4$ 30 mg/l, Na_2EDTA 45 mg/l, BA (6-benzylaminopurin) 0.5 mg/l, IBA 0.01 mg/l, inositol 100 mg/l, pH 6. After 4 weeks of cultivation in light the proliferating cultures were ready for the rooting to be monitored in *in vitro* conditions.

The apical segments of the shoots were 2 to 3 cm long; they were transferred into 370-ml glass flasks of 20 segments each, containing 60 ml of the cultivation medium MS (MURASHIGE, SKOOG 1962) and a concentration of growth regulators (IBA 0, 0.75, 1.5 mg/l and PP 333 0, 0.06, 0.12 mg/l), which were covered with a food sheet, and 10 segments each into 200 ml glass flasks containing 30 ml of the medium, which were closed with a metal stopper equipped with a septum adapted for gas collection. Each variant had 7 replications.

During rhizogenesis we evaluated the effect of the growth regulators in the medium on the number and length of arising adventitious roots and on the length of the shoot after 7 and 14 days of cultivation. Segments of bases (2 × 0.1 g) were taken on the day of the establishment of the experiment, then after 24 and 72 hours, and on the 7th and 14th days of cultivation to determine the level of ABA, and the production of ethylene, ethane

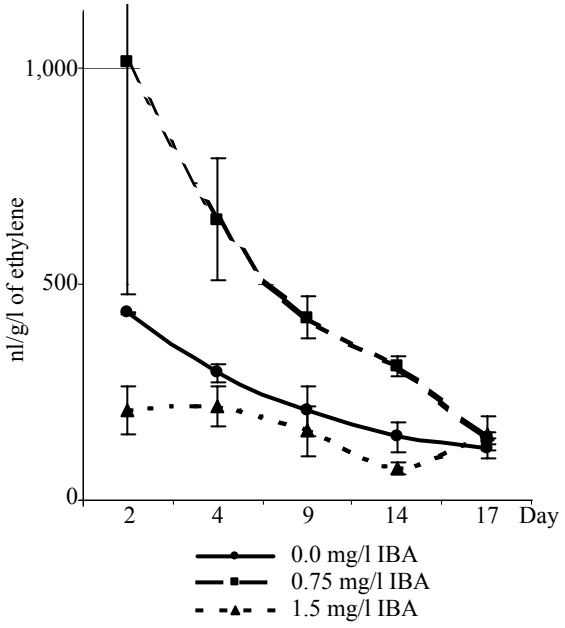

Fig. 1. The influence of IBA on ethylene production during the rooting period of Ishtara

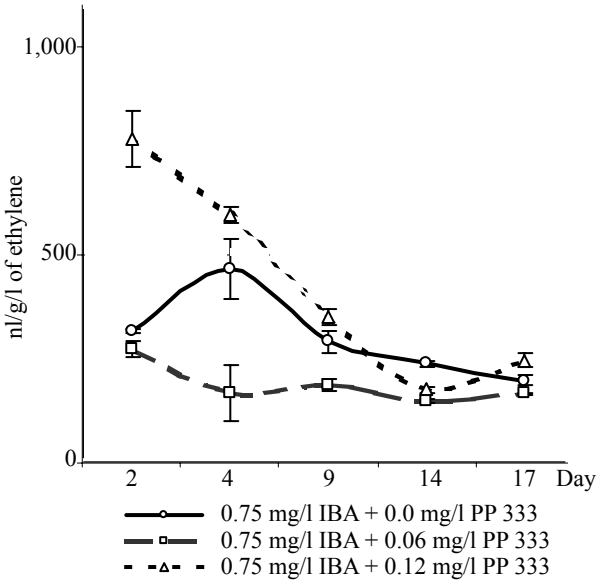

Fig. 2. The influence of IBA and PP 333 on ethylene production during the rooting period of Ishtara

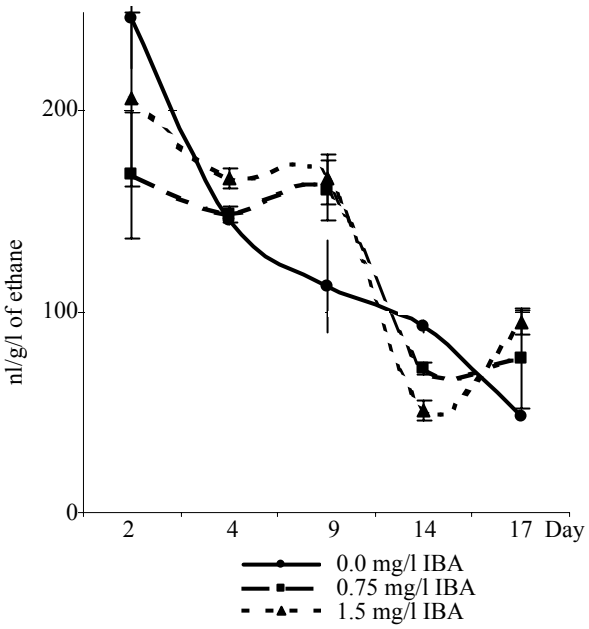

Fig. 3. The influence of IBA on ethane production during the rooting period of Ishtara

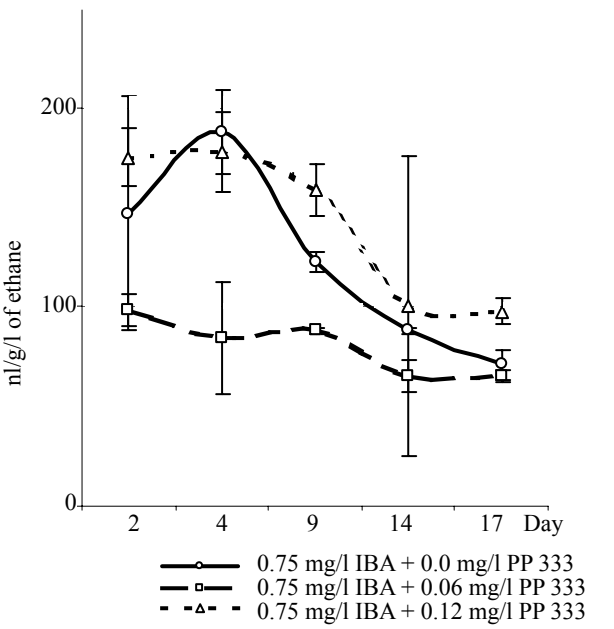

Fig. 4. The influence of IBA and PP 333 on ethane production during the rooting period of Ishtara

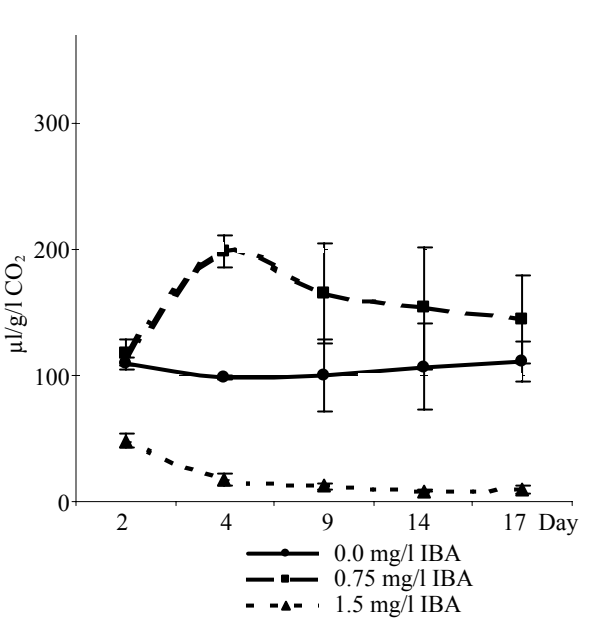

Fig. 5. The influence of IBA on CO_2 production during the rooting period of Ishtara

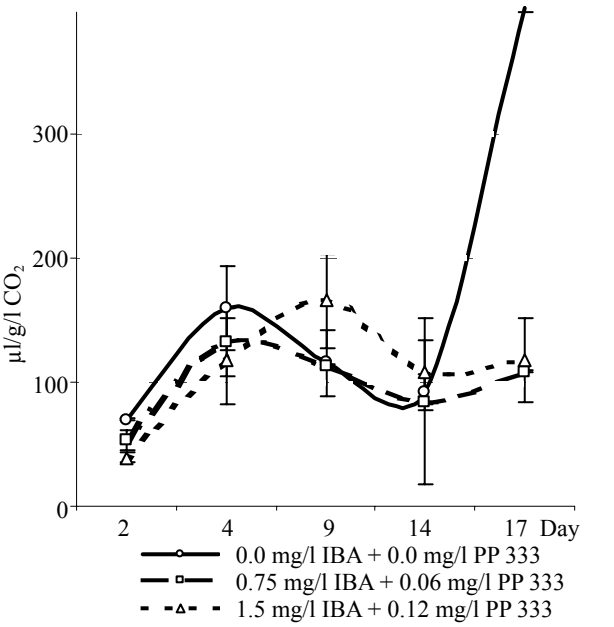

Fig. 6. The influence of IBA and PP 333 on CO_2 production during the rooting period of Ishtara

and CO_2 in the cultivation vessel. Cultivation proceeded at 25 ± 1°C under a light regime using fluorescent tubes (continual white fluorescent light – Tesla, 40W, 40 µmol/S/m^2) and a 16/8 hour photoperiod.

The Unistat programme (two-factor analysis, variation Anova) was used for statistical evaluations of the length of the aboveground part of the segment and of adventitious roots and their numbers. After conversion to the weight of the material and the gas standard, the ABA levels from 6 replications and the gas contents (7 replications) were averaged and the mean error was calculated. Figs. 1–12 show the results.

Determination of ethylene, ethane and carbon dioxide production

Within 24 and 72 hours of the establishment of the experiment and on the 7th and 14th day of cultivation, 2 × 1 ml of air was taken from the cultivation vessel closed with a rubber stopper with a tuberculin syringe. The air was analysed on a gas chromatograph of the firm FISSONS INSTRUMENT, Italy (50 m capillary column Al_2O_3 "S" 15 µm, ID 0.53 mm) where the temperature of the packing, column and detector for the assessment of gas hydrocarbons was

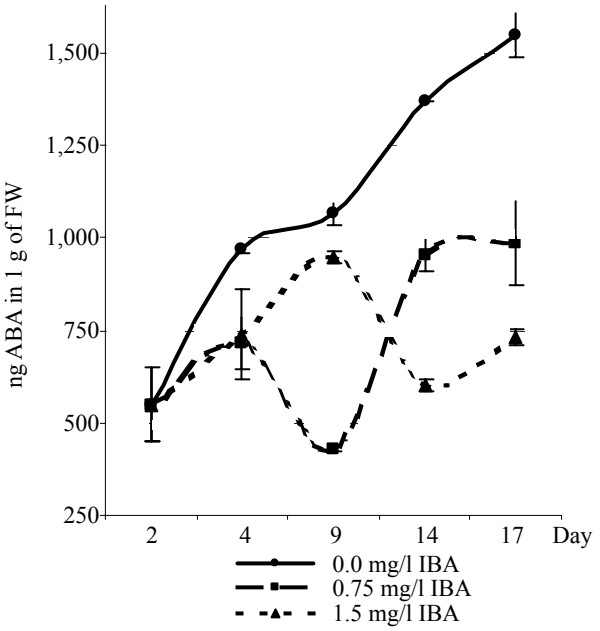

Fig. 7. The influence of IBA on content of ABA in the bases of shoots during the rooting period of Ishtara

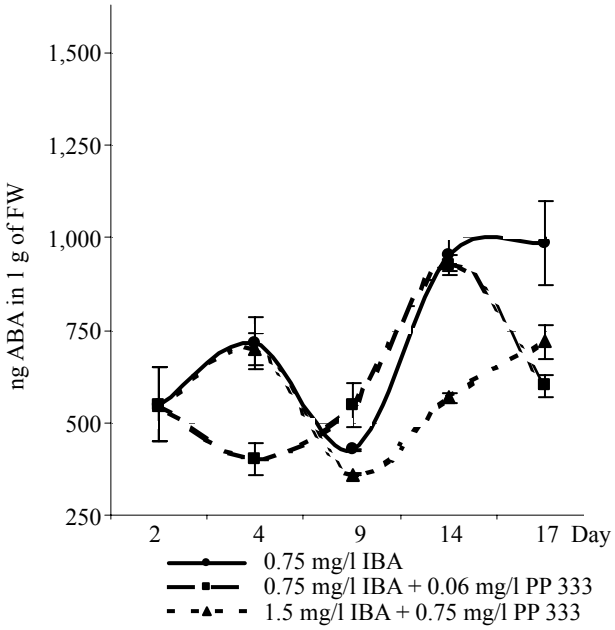

Fig. 8. The influence of IBA and PP 333 on ABA content in the bases of shoots during the rooting period of Ishtara

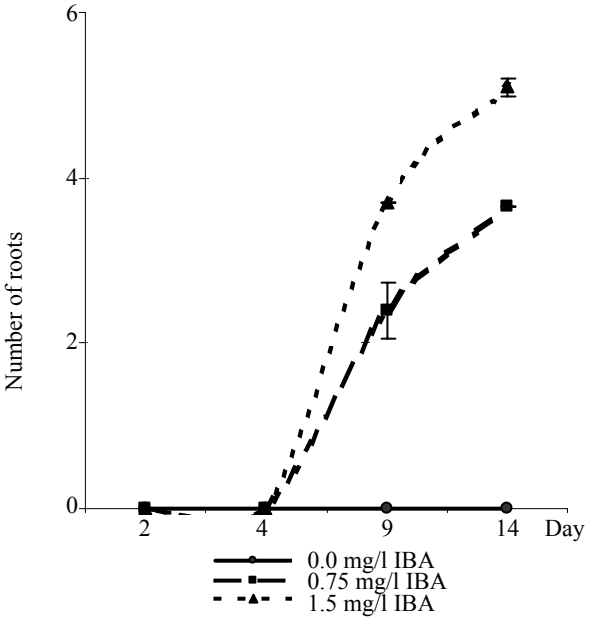

Fig. 9. The influence of IBA on the number of roots of Ishtara

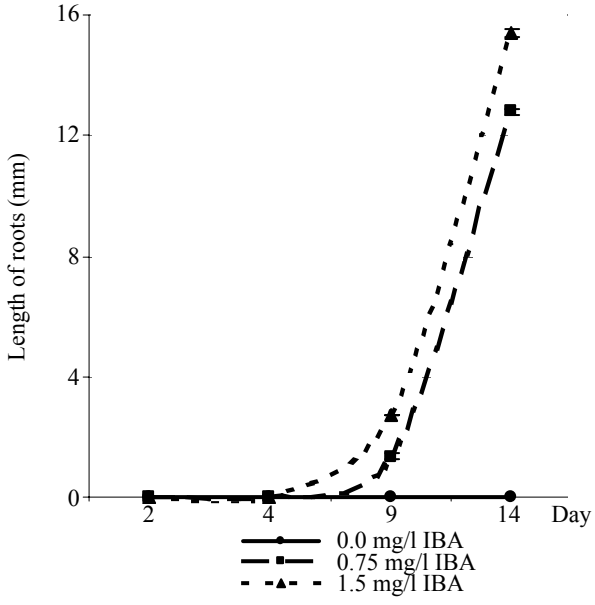

Fig. 10. The influence of IBA on the length of roots of Ishtara

230°C, 40°C and 200°C, respectively (FIŠEROVÁ, HRADILÍK 1994; FIŠEROVÁ et al. 2001), and on the CHROM 5, Praha – with a catharometer with a 1.5 m long column packed with PORAPAK Q to assess the level of carbon dioxide. Statistical evaluations were conducted after conversion to the ethylene and carbon dioxide standard in 1 ml of the air from the space where it was taken.

Radioimmunoassay (RIA) of the native abscisic acid (ABA)

Liquid nitrogen was poured over ca. 0.1 g of the base segments, then closed in a plastic case and left at –24°C in the freezer until processing according to the method of QUARRIE et al. (1988). RIA analysis was conducted using the monoclonal antibody MAC

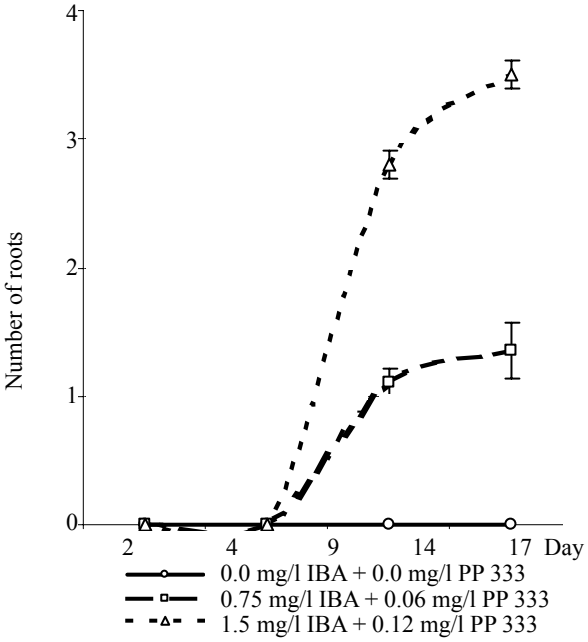

Fig. 11. The influence of IBA and PP 333 on the number of roots of Ishtara

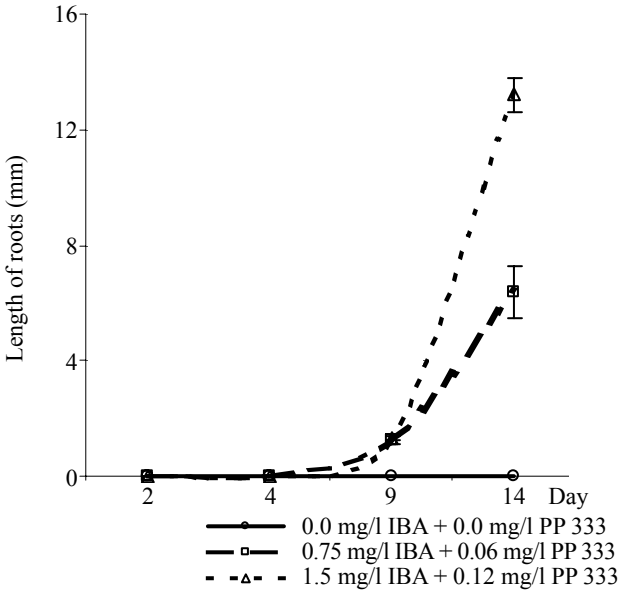

Fig. 12. The influence of IBA and PP 333 on the length of roots of Ishtara

252 (manufacturer Monoclonal Antibody Centre, Cambridge, UK) for the assessment of (+) – S – ABA and ^{3}H – ABA (Amersham, spec. radioact. 2.55 TBq/mmol, radioactive concentration 92.5 GBq/l). The activity of ^{3}H was measured with the scintillation spectrometer Packard 2,000 CA and the SECURIA – Packard programme.

RESULTS AND DISCUSSION

On the medium with 0.75 mg/l IBA the shoots produced statistically significantly higher levels of ethylene than the control shoots; a higher addition of IBA (1.5 mg/l) reduced ethylene production to a level lower than the control variant (without the IBA) – Fig. 1, but had the highest proportion of the longest roots (Fig. 11). RIOV and YANG (1989) reported that an application of IAA to the rooting mung bean cuttings inhibited the production of adventitious roots and the segments produced less ethylene than after the application of IBA, which stimulated the production of adventitious roots and ethylene alike and increased the number of roots but reduced their length. If AVG, the inhibitor of ethylene production, was applied, root formation declined. PSOTA et al. (1997) examined the production of adventitious roots in *Salix babylonica*, and they discovered that after the application of PAA (phenylacetic acid) the level of native auxin did not increase as compared to the control variant, but ethylene production increased as well as the number and length of the roots.

MARINO and VENTURA (1997) noticed that the accumulation of ethylene in the cultivation pot reduced the period of rhizogenesis of peach rootstock. The ethylene sorbent reduced the rooting % on the 9th day after the establishment of the experiment, but as soon as after 14 days there were no differences between the control and treated variants. On the 5th and 9th days the concentration of CO_2 was similar in all the variants; this fact leads us to the conclusion that the content of CO_2 does not affect root formation in peach rootstock. Nevertheless, from the 9th day the authors recommend aeration of the cultivation pots to prevent leaf yellowing.

If we applied IBA in our experiments, the CO_2 content increased for 4 days and then decreased. Higher concentrations of IBA resulted in the lowest production of CO_2 (Fig. 5).

MONCOUSIN et al. (1989) discovered that during the rooting of nodal segments of vine cuttings on a medium without growth regulators, the production of ethylene increased 2 hours after the establishment of the experiment (the values correspond to stress), then after 10 hours again, with a parallel increase in endogenous auxin, and before bud flushing again. FIŠEROVÁ and HRADILÍK (1994) also reported increased production of ethylene before the flushing of apical vine cuttings.

In explant rose cultures GASPAR (1990) observed that the ethylene level increased only on media with increasing levels of BA and IBA on the 4th and 8th days along with proliferation of the axillars. The growth activity of the plants was reduced in the presence of the ethylene inhibitor as well as after the application of exogenous ethylene; therefore the author lays emphasis on the importance of the action of native ethylene at the beginning of growth, but later aeration of the cultivation vessels is also necessary. During the rooting of nodal segments of cucumbers the production of ethylene and of roots was in direct proportion to the applied IBA

concentration in the medium. The addition of Ag + in the medium increased ethylene production even more, but reduced root formation, particularly when the concentration of IBA was low (HADID 1992). CLARK et al. (1999) conducted experiments with ethylene-insensitive plants (tomato) where the application of IBA did not initiate root formation, but an application of ACC (1-aminocyclopropan-1-carboxyl acid) did. Likewise, in experiments conducted by ROUT and DAS (1999) the addition of $AgNO_3$ tripled the regeneration of leaf explants of *Simarouba glauca* Linn.

In our experiments a lower concentration of IBA increased CO_2 production and as with ethylene, a higher concentration of IBA decreased CO_2 production below the level of the control variant (Fig. 5). In the paclobutrazol-enriched variants (Fig. 6), there were no statistically significant differences. Azalea segments rooted in a concentration of 1 mg/l CO_2 (BETTIN, FAUPEL 1990). RALLO and RIO (1990) also reported that the rooting ability of olive trees increased from 25 to 43% when the concentration of CO_2 increased from 0.2 to 0.8 mg/l to 0.4–1.8 mg/l. On the other hand, the axillary shoots of *Theobroma cacao* in *in vitro* conditions produced more roots only when cultivated in light and with 20 mg/l of CO_2 (FIGUERIA et al. 1991). Likewise OKADA (2003) reported that exogenous CO_2 increased shoot growth and rooting of prunus cuttings. According to our results, however, it is more likely that the production of CO_2 is in reverse proportion to the number and length of the roots (Figs. 5 and 6) and ranges between 9 and 180 mg/l CO_2.

IBA and paclobutrazol applications alike reduced the level of abscisic acid (Figs. 7 and 8) and this reduction is in reverse proportion to the number and length of the roots.

According to TARI and NAGY (1996) the applications of ABA and Ethrel abolish the inhibition of adventitious root formation in stem cuttings of paclobutrazol-treated bean. Paclobutrazol reduced root formation from 66 to 10%, however foliar spraying with ABA or ethrel increased rhizogenesis to 50%. Within 48 hours of ABA application the production of ethylene and the level of ACC in the base of the segments increased and ABA was able to increase root formation regardless of the effect of ethylene. Based on this finding the effect of paclobutrazol is not directly connected with gibberellin biosynthesis.

Figs. 3 and 4 show the changes in ethane production that decreases during cultivation as does ethylene. BLAKE et al. (1988) monitored the amount of ethane that in *Pseudotsuga menziesii* was in direct proportion with the disintegration of chlorophyll and in *Tsuga heterophylla* its production was very low.

In figures that are numbered from 7 to 12 the directly proportionate relationship between the content of abscisic acid and the formation of root system can be seen; in control variants of both series of experiments we can trace the increasing level of abscisic acid during cultivation that inhibits the formation of the root system.

CONCLUSION

Not only auxin is important during rooting but also the content of paclobutrazol with a modified formula of the MS medium, as well as the light conditions and the temperature; also the preparation of the shoots for rooting by changing the content of plant hormones during the last "multiplication" subculture. A significant factor may also be the period of the cultivation and the overall duration of the cultivation of the culture. In our experiments a lower concentration of IBA increased CO_2 production and as with ethylene, a higher concentration of IBA decreased CO_2 production below the level of the control variant. In the paclobutrazol-enriched variants, there were no statistically significant differences. IBA and paclobutrazol applications alike reduced the level of abscisic acid and this reduction was in reverse proportion to the number and length of the roots.

The achieved results confirm the positive effect of the IBA concentration, and the combination of IBA with paclobutrazol, on the rooting of Ishtara shoots, as in other tree species (ORLIKOWSKA 1992; FRANC 1998).

An addition of 1.5 mg/l IBA stimulates the formation of adventitious roots of the Ishtara shoots, similarly like the interactive effect of IBA (concentration of 0.75 mg/l) and PP 333 (0.12 mg/l) at the same time reducing the production of ethylene and carbon dioxide in the shoots and abscisic acid in the base of the shoots.

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Vliv růstových regulátorů na zakořeňování prýtů broskvoňové podnože Ishtara v podmínkách *in vitro*

ABSTRAKT: Cílem práce bylo sledovat vliv růstových regulátorů indolyl-3-máselné kyseliny (IBA) a paclobutrazolu (PP 333) na zakořeňování prýtů broskvoňové podnože Ishtara na světle v podmínkách *in vitro*. V první fázi pokusů byl sledován vliv koncentrace IBA (0, 0,75, 1,5 mg/l) a v druhé fázi pokusů vliv interakce IBA (0,75 mg/l) a PP 333 (0,06 a 0,12 mg/l) v modifikovaném médiu MS optimálním pro zakořeňování broskvoňové podnože Ishtara. V průběhu kultivace prýtů byla hodnocena tvorba kořenů (průměrná délka a počet) a výška prýtů. Současně byla v procesu rhizogeneze sledována produkce etylenu, etanu a CO_2 prýty v podmínkách *in vitro* a hladina ABA v bázi prýtu. Největší počet nejdelších kořenů byl při ošetření 1,5 mg/l IBA a kultivované segmenty produkovaly nejméně etylenu, CO_2 a v bázích byl nejnižší obsah ABA. Obsah abscisové kyseliny byl snižován jak aplikací IBA, tak aplikací PP 333 a její obsah byl nepřímo úměrný počtu a délce kořenů.

Klíčová slova: IBA; PP 333; etylen; ABA; *Prunus persica* L.; zakořeňování *in vitro*

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