The effects of cytokinin types and their concentration on *in vitro* multiplication of sweet cherry cv. Lapins (*Prunus avium* L.)

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ABSTRACT: Determination of the most optimal types and concentrations of plant growth regulators as medium constituents is one of the most important aspects of successful micropropagation, among other *in vitro* factors. With the aim of optimization of *in vitro* multiplication of sweet cherry cv. Lapins the effect of following cytokinins has been studied: benzyladenine (BA), isopentenyl adenine (2iP), kinetin (KIN) and thidiazuron (TDZ) at concentrations of 1, 2, 5, 10 and 15μM, combined with auxine, indole-3-butyric acid (IBA) at concentrations of 0, 0.5, 2.5 and 5μM. Murashige and Skoog (1962) was the basic medium used in all the combinations. The following multiplication parameters were monitored: multiplication index, length of axial and lateral shoots. Fresh and dry shoot weight (callus, stem and leaves – S + L) were determined. Some specific issues, such as colour, leaf and callus size, leaf roll, incidence of chlorosis or necrosis along with occurrence of rhizogenesis, i.e. roots unusual for this phase of micropropagation, were also monitored. The highest multiplication index as well as length of axial and lateral shoots was obtained on media with BA. Very poor multiplication, with large sized shoots and big leaves, was achieved on media with 2iP, TDZ and KIN, whereas in many combinations with 2iP, and particularly in those with KIN, rhizogenesis was induced. Obtained results suggest that the choice of cytokinins for the phase of multiplication of sweet cherry is limited to BA. For more rapid micropropagation, through joining rooting and multiplication phases, KIN and 2iP may be applied. The latter two may be also used to obtain sturdy shoots (elongation phase, prior to rooting).

Keywords: cytokinins; *Prunus avium* L.; *in vitro* multiplication; root induction

Sweet cherry cv. Lapins (*Prunus avium* L.) is one of the leading sweet cherry cultivars of the world assortment, also known as Cherokee or 'self-fertile Bing'. It is a popular, self-fertile dessert cherry, developed at the Canadian Fruit Research Station, Summerland, British Columbia, singled out from the progeny of parental combination of Van × Stella (Kappel et al. 2003). The tree is vigorous, of erect habitus and excellent cropping. The fruits are roundish-cordate in shape, with purple red ground skin colour. It is partly resistant to cracking. This dwarf cherry is ideal for space-limited plantings.

As this sweet cherry cultivar has excellent properties and rapid changes in the assortment on the market are trend, micropropagation is obviously an ideal method of rapid propagation of this cultivar and its subsequent introduction into production.

It is well known that cytokinins promote cell division and cell expansion in plant tissue culture and many studies have reported suitable cytokinin types and their concentrations for each species.

Hence, the objective of this study was to develop a protocol for propagation of this sweet cherry cultivar as well as to determine the most optimal conditions for multiplication phase with the use of 4 cytokinins (BA, TDZ, 2iP and KIN), particularly combined with IBA.

BA is widely used in micropropagation of stone fruits and has given good results so far when combined with different auxines (Ružić et al. 1984, 2000, 2001; Ružić, Cerović 1985, 1990; Cerović, Ružić 1987).

Usually, extremely low concentrations of TDZ are needed to stimulate axillary shoot proliferation (Huetteman, Preece 1993). Investigations of TDZ reached their climax in the 1990s: adventitious

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shoots were regenerated from cotyledons of plum *Prunus domestica* L., sour cherry *Prunus cerasus* and peach *Prunus persica* L. (Mante et al. 1989), *Rubus* sp. (Fiola et al. 1990; Cousineau, Donnelly 1991); stimulation of organogenesis from leaf in *Rubus* and *Malus domestica* L. (Fasolo et al. 1988; Swartz et al. 1990), *Pyrus* sp. (Chevreau et al. 1989).

Cytokinins 2iP and KIN are rarely used in micropropagation of fruit varieties (Ružić, Cerović 1985; Jaakola et al. 2001; Ostrolucká et al. 2004; Góralski et al. 2005), hence the investigation of their influence on multiplication of sweet cherry cv. Lapins helped to achieve the projected goal.

MATERIAL AND METHODS

Plant material

Due to its current status in fruit growing and increasing spread, the sweet cherry cv. Lapins was used in this study as a model plant. The explants were isolated from the lateral buds, underwent surface sterilization (it was kept for an hour and half under running water, 1 minute in 70% ethanol, 10 min in sodium hypochlorite and rinsed 3 times in sterile water) and placed on the regeneration medium. To avoid the effect of the residues from the previous medium, shoots were sub-cultured, prior to the placement on the appropriate media, so that the morphometric measurements and samples for fresh/dry weight were taken from the second subculture. Six culture vessels × 3 uniform shoots × 80 treatments (20 media/combinations × 4 cytokinins) × 3 replications were used.

Media

The cultures were grown on regeneration medium Murashige and Skoog (1962) (MS) with (in μM): benzyladenine (BA) 8.9, indole-3-butyric acid (IBA) 2.5 and gibberellic acid (GA_3) 0.3, and upon establishment of aseptic culture the shoots were multiplied on MS medium with (in μM): BA 4.4, IBA 0.5 and GA_3 0.3. Upon multiplication of sufficient number of shoots they were placed on the MS medium with: BA, isopentenyl adenine (2iP), kinetin (KIN) and

Table 1. Multiplication *in vitro* of sweet cherry cv. Lapins on media with BAP

No. of media	BAP (μM)	IBA (μM)	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
B1	1	0.0	1.78 de*	1.41 fghi	0.67 bcde
B2	1	0.5	2.17 bcd	1.82 abc	0.72 abc
B3	1	2.5	2.33 abc	1.71 abcd	0.87 a
B4	1	5.0	1.65 def	1.20 jk	0.67 bcde
B5	2	0.0	2.17 bcd	1.38 ghij	0.67 bcde
B6	2	0.5	2.39 ab	1.82 abc	0.82 ab
B7	2	2.5	1.83 cde	1.63 bcdef	0.65 bcde
B8	2	5.0	2.56 ab	1.53 defgh	0.71 abc
B9	5	0.0	1.50 efg	1.65 bcde	0.53 de
B10	5	0.5	2.83 a	1.60 cdefg	0.63 cde
B11	5	2.5	1.83 cde	1.93 a	0.69 bcd
B12	5	5.0	2.39 ab	1.84 ab	0.85 a
B13	10	0.0	1.00 g	1.18 jk	–
B14	10	0.5	1.06 g	1.13 k	–
B15	10	2.5	1.50 efg	1.32 hijk	0.57 cde
B16	10	5.0	1.22 fg	1.44 efghi	0.55 de
B17	15	0.0	1.16 fg	1.27 ijk	0.52 e
B18	15	0.5	1.12 g	1.26 ijk	0.55 de
B19	15	2.5	2.17 bcd	1.20 jk	0.52 de
B20	15	5.0	1.50 efg	1.39 ghij	0.56 de

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's multiple range test

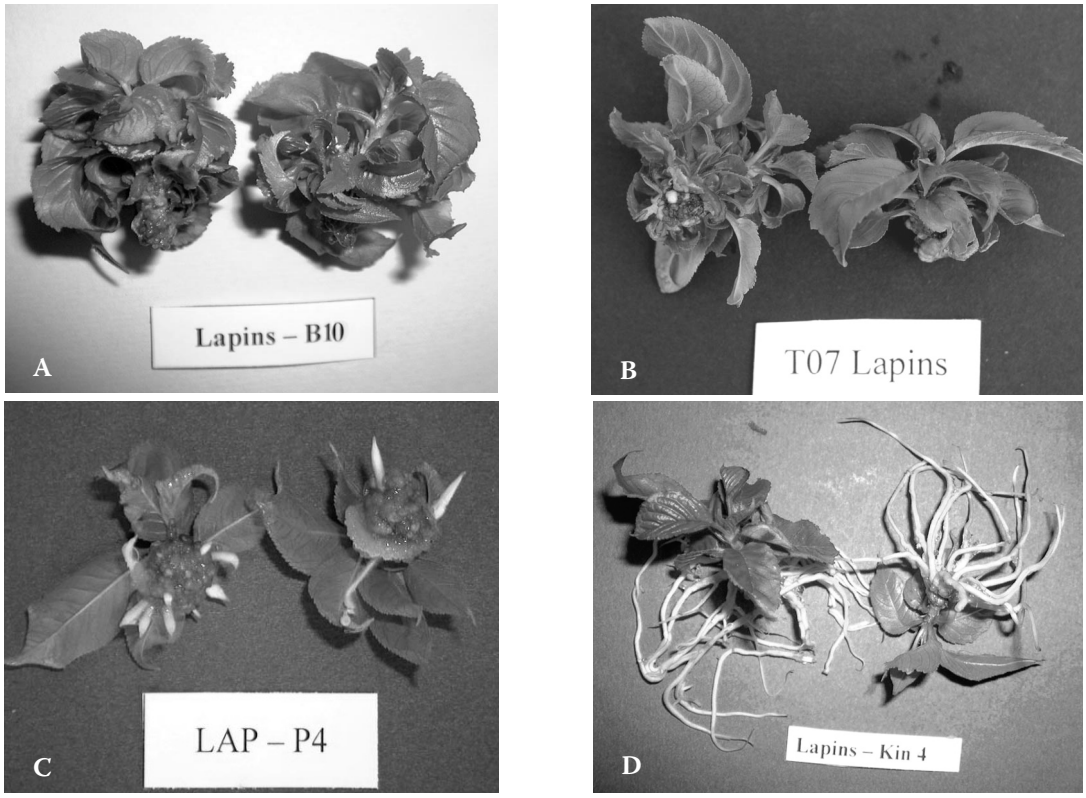

Fig. 1. Shoots of cv. Lapins on medium with: A – 5μM BAP + 0.5μM IBA, B – 2μM TDZ + 2.5μM IBA, C – 1μM 2iP + 5μM IBA, D – 2μM KIN + 5μM IBA

thidiazuron (TDZ) at the concentrations of 1, 2, 5, 10 and 15μM, combined with auxine, IBA at concentrations of 0, 0.5, 2.5 and 5μM. Stock solution of TDZ was obtained through its dissolving with DMSO and sterilized by autoclaving. Prior to autoclaving, the pH value of all the media was adjusted to 5.75 with 0.1N KOH. The media were sterilized in an autoclave for 20 min at 120°C. All the media contained agar at the concentration of 7 g/l and 20 g/l of sucrose.

Multiplication parameters

Multiplication parameters were determined by the usage of 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. Fresh and dry shoot weight (callus, stem and leaves) were further determined. Data are reported as total weight of the whole shoot (leaves + stem – S + L) plus callus. Upon removal from the medium shoots were washed in distilled water, dried with filter paper and their FW was determined. For the DW, shoots were dried in oven at 65–70°C for 48 h.

Some specific issues, such as colour, leaf and callus size, leaf roll, incidence of chlorosis or necrosis along with occurence of rhizogenesis, i.e. roots, unusual for this phase of micropropagation, were also monitored.

Cultural conditions

The cultures were grown under a 16 h photoperiod, with a light intensity of 41 mol/m^2/s on the culture surface provided with cool white fluorescent tubes 40 W, 6,500°K. The temperature was 25 ± 1°C.

Data analysis

The data were analyzed by ANOVA and *F*-test, as well as by individual Duncan's multiple range test.

RESULTS

Cytokinins are classified into two major groups (by their chemical structures): synthetic phenylurea derivates, and adenine derivates, which may occur naturally. The effect of these two groups of cytokinins was studied in this paper.

Aseptic culture and regeneration of shoots was obtained from axillary buds in 35% of explants, whereas 65% of explants were tarnished or infected.

The best multiplication parameters with BAP cytokinin were obtained with the concentration of

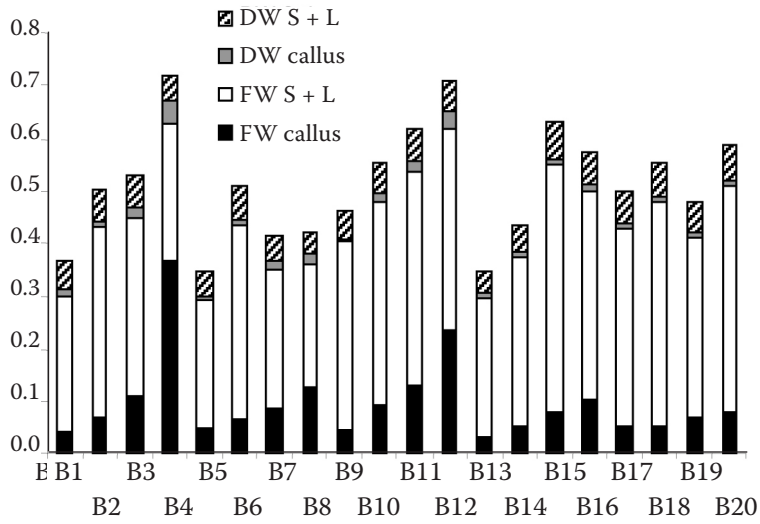

Fig. 2. Fresh and dry shoot weight of cv. Lapins (g) on media with BAP

5μM with 0.5 variation of IBA for multiplication index, up to 2.5μM for the shoot length (Table 1). On the medium with 5μM BAP and 0.5μM IBA, which gave the highest multiplication index, the plants were big, with dark green leaves and their calli were dark green to brown, with firm, nodular consistency (Fig. 1A). On the other media with BAP, obtained plants were mainly large which also influenced considerable FW and DW of shoots, particularly at higher concentrations. As for the callus weight, the linear increase was recorded with rise in IBA concentration (Fig. 2).

On the media with TDZ, multiplication index did not exceed 1.5 (Table 2), but fresh and dry shoot weight were higher, especially for callus. The linear increase of callus weight was recorded with rise in IBA concentration as well (Fig. 3). No multiplication occurred on medium with 5, 10 and 15μM and

Table 2. Multiplication *in vitro* of sweet cherry cv. Lapins on media with TDZ

No. of media	TDZ (μM)	IBA (μM)	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
T01	1	0.0	1.00 b	1.01 cdef	–
T02	1	0.5	1.20 ab	1.18 abc	0.55 b
T03	1	2.5	1.30 a	1.32 a	0.50 b
T04	1	5.0	1.40 a	1.25 ab	0.53 b
T05	2	0.0	1.40 a	1.25 ab	0.68 a
T06	2	0.5	1.25 ab	1.12 abcde	0.53 b
T07	2	2.5	1.50 a	1.03 cdef	0.52 b
T08	2	5.0	1.40 a	1.11 bcde	0.55 b
T1	5	0.0	1.00 b	1.00 cdef	–
T2	5	0.5	1.00 b	0.86 f	–
T3	5	2.5	1.00 b	0.95 def	–
T4	5	5.0	1.00 b	0.95 ef	–
T5	10	0.0	1.00 b	1.16 abcd	–
T6	10	0.5	1.00 b	0.88 f	–
T7	10	2.5	1.00 b	0.83 f	–
T8	10	5.0	1.00 b	0.92 ef	–
T9	15	0.0	1.05 b	0.83 f	–
T10	15	0.5	1.00 b	0.98 cdef	–
T11	15	2.5	1.05 b	0.97 def	–
T12	15	5.0	1.00 b	1.09 bcde	–

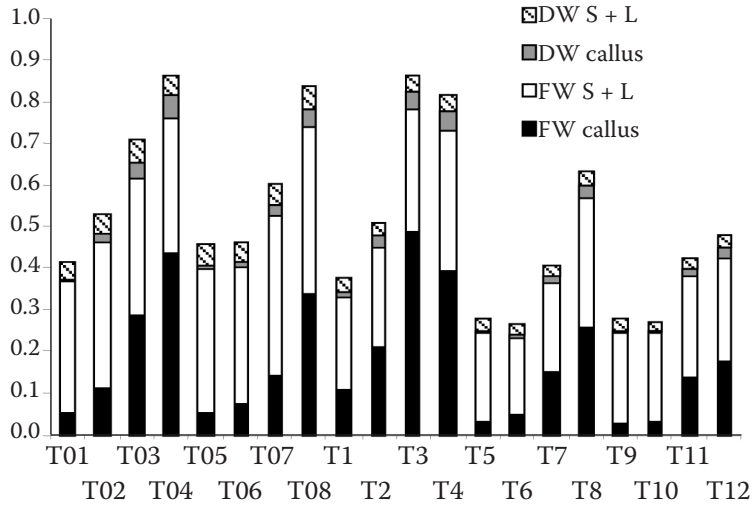

Fig. 3. Fresh and dry shoot weight of cv. Lapins (g) on media with TDZ

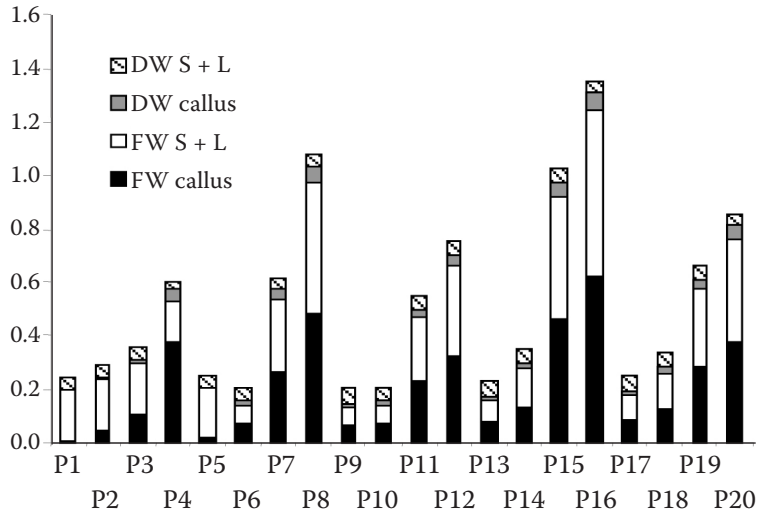

Fig. 4. Fresh and dry shoot weight of cv. Lapins (g) on media with 2iP

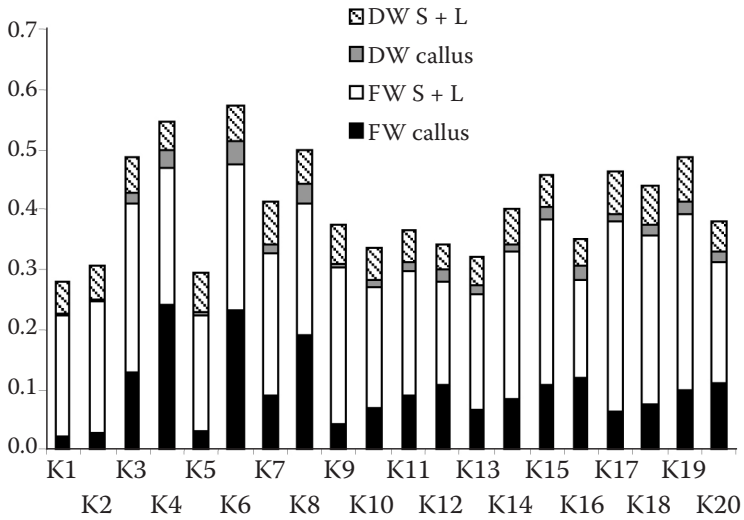

Fig. 5. Fresh and dry shoot weight of cv. Lapins (g) on media with KIN

obtained shoots had substantially lower values (Table 2). Our results suggest the following: shoots are shortened, which is largely a basic characteristic; the stem is strong, with large leaves and firm, nodular callus (Fig. 1B).

The properties of plants grown on media with 2iP are as follows: very large, wide leaves and strong shoots without multiplication (Table 3), and on media with 1μM 2iP, particularly combined with 5μM IBA, the rooting percentage amounted even up to 72.22% (Ta-

Table 3. Multiplication *in vitro* of sweet cherry cv. Lapins on media with 2iP

No. of media	2iP (μM)	IBA (μM)	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
P1	1	0.0	1.00 b	1.34 abc	–
P2	1	0.5	1.00 b	1.23 abcde	–
P3	1	2.5	1.00 b	1.13 cde	–
P4	1	5.0	1.00 b	1.01 e	–
P5	2	0.0	1.00 b	1.24 abcde	–
P6	2	0.5	1.10 ab	1.05 de	0.40 b
P7	2	2.5	1.00 b	1.18 bcde	–
P8	2	5.0	1.00 b	1.18 bcde	–
P9	5	0.0	1.00 b	1.48 a	–
P10	5	0.5	1.00 b	1.21 bcde	–
P11	5	2.5	1.00 b	1.17 cde	–
P12	5	5.0	1.00 b	1.26 abcde	–
P13	10	0.0	1.00 b	1.40 ab	–
P14	10	0.5	1.00 b	1.31 abc	–
P15	10	2.5	1.00 b	1.37 abc	–
P16	10	5.0	1.05 b	1.21 bcde	–
P17	15	0.0	1.17 a	1.48 a	0.55 a
P18	15	0.5	1.00 b	1.27 abcd	–
P19	15	2.5	1.00 b	1.22 abcde	–
P20	15	5.0	1.05 b	1.35 abc	–

ble 4, Fig. 1C). The linear increase of callus weight was also recorded with rise in IBA concentration (Fig. 4).

On the media with KIN the shoots were large with big leaves and small shootlets at the shoot base (up to 0.3 cm) and brown coloured, compact callus. The highest stem weight did not exceed the one obtained on media with BAP, 2iP and TDZ (Fig. 5). The linear increase of callus weight was mainly recorded with rise in IBA concentration (Fig. 5). Length of axial shoots was the highest precisely on those media with KIN where rhizogenesis occurred (1μM KIN and 5μM IBA), even up to 100% of rooted plants (Tables 5 and 6). Occurrence of rooting was observed on media that contained up to 5μM KIN (except for media with 2.5 and 5μM IBA). The plants had exceptionally large, dark green leaves and well developed, white, radial root system (Fig. 1D).

DISCUSSION

On the basis of the obtained results it may be concluded that there are differences in uptake of cytokinins, recognition by the cells, or mechanisms of action of the cytokinin compounds. However, cytokinins in our experiment can be divided into two groups: very active group – only BA, which was

Table 4. Parameters of the rooted shoots grown on media with 2iP

No. of media	Percentage of rooting	Average number of roots per rooted plant	Average length of roots per rooted plant	Average fresh root weight (g)	Average dry root weight (g)
P3	27.78	2.8 b	0.88 b	0.028 ab	0.0026 ab
P4	72.22	4.9 a	0.94 b	0.042 a	0.0032 a
P6	5.56	2.0 b	1.10 ab	0.010 b	0.0008 ab
P7	5.56	1.0 b	1.00 ab	0.010 b	0.0010 ab
P8	11.11	3.0 ab	0.85 b	0.030 ab	0.0023 ab
P12	5.56	1.0 b	1.30 a	0.010 b	0.0006 b

Table 5. Multiplication *in vitro* of sweet cherry cv. Lapins on media with KIN

No. of media	KIN (μM)	IBA (μM)	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)
KIN1	1	0.0	1.00 b	1.47 a	–
KIN2	1	0.5	1.00 b	1.39 a	–
KIN3	1	2.5	1.00 b	1.43 a	–
KIN4	1	5.0	1.00 b	1.55 a	–
KIN5	2	0.0	1.00 b	1.45 a	–
KIN6	2	0.5	1.00 b	1.22 b	–
KIN7	2	2.5	1.00 b	1.43 a	–
KIN8	2	5.0	1.00 b	1.24 b	–
KIN9	5	0.0	1.00 b	1.20 b	–
KIN10	5	0.5	1.00 b	1.16 bc	–
KIN11	5	2.5	1.00 b	1.23 b	–
KIN12	5	5.0	1.00 b	1.11 bc	–
KIN13	10	0.0	1.06 b	1.16 bc	–
KIN14	10	0.5	1.17 b	1.22 b	0.50 c
KIN15	10	2.5	1.17 b	1.16 bc	0.50 c
KIN16	10	5.0	1.06 b	1.04 cd	–
KIN17	15	0.0	1.50 a	1.15 bc	0.57 b
KIN18	15	0.5	1.61 a	1.16 bc	0.52 c
KIN19	15	2.5	1.11 b	1.14 bc	0.63 a
KIN20	15	5.0	1.06 b	0.91 d	–

more effective, i.e. more shoots of cherry cv. Lapins formed, whereas TDZ, 2iP and KIN exhibited rather weak effects on multiplication. These results agree with results obtained by Kadota and Niimi (2003) with pear *Pyrus pyrifolia* N.; they suggest that BAP displayed more noticeable effect than TDZ and kinetin, i.e. BA is more suitable for shoot multiplication of pear than phenylurea derivatives. It is well known that high concentrations of cytokinins of adenine type are often necessary for growth and differentiation of tissue culture.

However, according to Mok et al. (1987) the reason for the high activity of low concentrations of TDZ in woody plant micropropagation was not investigated at a molecular level. It is a known fact that TDZ stimulates endogenous biosynthesis of cytokinins, which brings about an increase in the level of naturally occurring cytokinins, and it is likely to have a

Table 6. Parameters of the rooted shoots grown on media with KIN

No. of media	Percentage of rooting	Average number of roots per rooted plant	Average length of roots per rooted plant	Average fresh root weight (g)	Average dry root weight (g)
KIN1	44.44	3.9 cd	1.87 bc	0.044 c	0.0052 cd
KIN2	94.44	5.6 bcd	1.54 bcd	0.070 c	0.0083 cd
KIN3	94.44	9.2 ab	2.90 a	0.310 a	0.0270 b
KIN4	100.00	12.3 a	3.10 a	0.390 a	0.0370 a
KIN5	55.56	4.6 bcd	2.15 b	0.034 c	0.0077 cd
KIN6	94.44	10.4 a	1.01 d	0.100 bc	0.0109 c
KIN7	77.78	12.7 a	1.69 bcd	0.189 b	0.0202 b
KIN8	100.00	8.4 abc	1.23 cd	0.109 bc	0.0129 c
KIN9	11.11	1.5 d	1.93 bc	0.015 c	0.0023 d
KIN10	33.33	2.5 d	2.11 b	0.086 bc	0.0104 c

common site of action with the naturally occurring cytokinins. In general, TDZ increases shoot formation of many woody plant species more efficiently than purine adenine derivatives, but it is ineffective for the proliferation in some species (Huetteman, Preece 1993). TDZ at used concentrations has not induced expected rise of multiplication level in cv. Lapins.

On the other hand, TDZ may inhibit shoot elongation (Huetteman, Preece 1993) and it induces formation of shortened internodes in apple cv. Gala (van Nieuwkerk et al. 1986). In the present work, TDZ has also an effect on reduction of multiplied shoot length of cv. Lapins, especially with the concentrations of 5–15μM. The results obtained with *L. corniculatus* shoots seedling showed that even in the lowest concentrations (0.08 and 0.22μM), TDZ retarded elongation (Nikolić et al. 2006). Similar responses were reported by a number of researchers with several woody species (Thengane et al. 2001; Pruski et al. 2005, etc).

Occurrence of fasciation or hyperhydricity, typical for TDZ, was not recorded, although Kadota and Niimi (2003) concluded that hyperhydricity in explants of *Pyrus pyrifolia* is more affected by TDZ than by BA or KIN.

However, in some other species, such as endemic species *Decaleptis Hamiltonii*, the best multiplication was obtained with 2iP, in comparison with KIN, BAP and TDZ (Giridhar et al. 2005). Optimal growth of *Davidsonia jerseyana* was also recorded with 0.01μM 2iP on medium, compared with BA (Nand et al. 2004). Ostrolucká et al. (2004) found that 2iP plays a significant part in regeneration of *Vaccinium* species, which points to the genetic specificity of species towards growth regulators. Besides very long axial shoots obtained in some combinations (5μM, 10 and 15μM 2iP) along with high callus and shoot weight obtained, this cytokinin had no positive effect on sweet cherry cv. Lapins.

Cytokinin KIN mainly influenced shoot growth of cherry cv. Lapins, whereas it made little impact on multiplication. Some species, such as *Tabernaemontana fuschsiaefolia* L. (Apocynaceae), exhibited higher multiplication rate on media with KIN than BAP (de Oliveira et al. 2003).

It is well known that rooting of excised microshoots may be difficult because of a carry-over effect from cytokinins in the shoot proliferation medium especially using a cytokinin as potent as TDZ. In our experiment we have observed some unusual behaviour, i.e. rooting in multiplication phase with KIN and 2iP. Occurrence of rooting during multiplication phase on media with KIN, even when it is not combined with auxines, is very rare in fruit varieties. However, in species Boston fern the highest rate of rooting was obtained exactly on media that contained only kinetin at concentration of 6μM (Beck, Caponetti 1983). Christianson and Warnick (1983) postulated that the competence for a given organ can be channeled or changed into another type of competence by means of an appropriate auxin:cytokinin ratio.

CONCLUSION

Thus, analysis of the effect of all 4 cytokinins on multiplication phase of sweet cherry infers that BAP gives the best results.

For more rapid micropropagation, through joining rooting and multiplication phases due to occurrence of rhizogenesis, KIN and 2iP may be applied. For obtainment of sturdy and long shoots (elongation phase, prior to rooting) KIN and 2iP may also be used. TDZ is being selected for micropropagation of wide array of woody species because of tremendous ability to stimulate shoot proliferation but it does not stimulate axillary shoot proliferation of cherry cv. Lapins.

Obtained results undoubtedly suggest that the cytokinins type and concentration suitable for micropropagation of woody plants may depend on plant species, i.e. are probably genotype-dependent.

Our further investigation will be based on the study of the effect of combination of two cytokinins, in order to enhance both multiplication index and shoot quality for further sub-cultivation aimed at economic and rapid introduction of this sweet cherry cultivar into production.

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Vliv různých typů cytokininů a jejich koncentrace na množení třešně odrůdy Lapins (*Prunus avium* L.) v podmínkách *in vitro*

ABSTRAKT: Jedním z nejdůležitějších faktorů ovlivňujících úspěch mikrorozmnožování *in vitro* je určení optimálního zastoupení růstových regulátorů, které jsou složkami kultivačního média, a jejich koncentrací. Cílem práce byla optimalizace množení třešně odrůdy Lapins *in vitro*. Byl studován vliv následujících cytokininů: benzyladenin (BA), isopentenyl adenin (2iP), kinetin (KIN) a thidiazuron (TDZ) v koncentracích 1, 2, 5, 10 a 15μM v kombinaci s auxinem, kyselinou 3-indolylmáselnou (IBA) v koncentracích 0, 0,5, 2,5 a 5μM. Bazálním médiem použitým ve všech kombinacích růstových regulátorů bylo médium Murashige a Skoog (1962). Byly monitorovány následující parametry: index množení, délky axiálních a postranních výhonů. Dále byly stanoveny hmotnosti čerstvé a suché hmoty výhonů (kalus, stonek a listy – S + L). Byly rovněž sledovány některé další specifické charakteristiky, jako je barva, velikost listu a kalusu, výskyt svinutých listů, chlorózy nebo nekrózy společně s výskytem rizogeneze, neobvyklé pro tuto fázi mikrorozmnožování. Nejvyššího indexu množení (stejně jako největší délky axiálních a laterálních výhonů) bylo dosaženo na médiu obsahujícím benzyladenin (BA). Velmi špatná multiplikace doprovázená dlouhými výhony a velkými listy byla zaznamenána na médiích s 2iP, TDZ a KIN, zatímco v mnoha použitých kombinacích s 2iP a KIN byla indukována rizogeneze. Dosažené výsledky naznačují, že pro tuto fázi množení třešní se jako jediný vhodný cytokinin jeví BA. Pro urychlení mikrorozmnožování pomocí současné indukce zakořeňování při množení mohou být aplikovány KIN a 2iP. Oba tyto cytokininy mohou být také použity pro získání robustních výhonů (prodlužující fáze, předcházející zakořeňování).

Klíčová slova: cytokininy; *Prunus avium* L.; *in vitro* množení; indukce tvorby kořenů

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