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UPTAKE AND METABOLISM OF 2,4-DICHLOROPHENOXYACETIC ACID DURING INDUCTION OF NORWAY SPRUCE (*PICEA ABIES* (L.) KARST.) SOMATIC EMBRYOGENESIS

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Abstract

VLAŠÍNOVÁ, H., HAVEL, L., KLEMŠ, M., PROCHÁZKA, S.: *Uptake and metabolism of 2,4-dichlorophenoxyacetic acid during induction of Norway spruce (Picea abies (L.) Karst.) somatic embryogenesis*. Acta univ. agric. et silvic. Mendel. Brun., 2005, LIII, No. 5, pp. 169–174

Uptake and metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) into zygotic embryos of Norway spruce (*Picea abies* (L.) Karst.) were studied on medium used for induction of somatic embryogenesis. The main task of this work was to study effect of longitudinal bisection of embryos, which was found as increasing the subsequent induction of embryogenic cultures. The maximal uptake of 2,4-D per one embryo was detected after 16 hours on medium in bisected embryos. The bisection increased 2,4-D uptake per embryo in first 16th hours, but then increased its release back to the medium. The metabolism of 2,4-D in bisected embryos was lower than in intact ones during first two days of culture.

2,4-dichlorophenoxyacetic acid, Norway spruce, somatic embryogenesis

Auxins play an important role in many aspects of plant growth and differentiation. Both exogenous and endogenous auxins are closely involved in the process of somatic embryogenesis (Michalczuk et al., 1992). Among different auxins 2,4-dichlorophenoxyacetic acid (2,4-D) was found as most commonly applied in somatic embryogenesis induction (Attree and Fowke, 1993).

In conifers, where the general patterns of somatic embryogenesis are similar in many species, the zygotic embryos (ZEs) in different phases of their development were mostly used as primary explants (c.f. Jain et al., 1995). In Norway spruce the embryogenic culture can be initiated from hypocotyl, or cotyledons with shoot apex of ZEs (Mo and von Arnold, 1991).

We studied uptake and transport of 2,4-D into intact and bisected ripe ZEs of Norway spruce and appearance of its biologically active and inactive metabolites during the induction of embryogenic culture. Free 2,4-D and also its non-polar metabolites are considered physiologically active while polar forms of 2,4-D metabolism are known as inactive (Crafts, 1953).

Even if the first embryogenic cultures in Norway spruce were initiated many years ago (Chalupa, 1985; Hakman and von Arnold, 1985) the details of action of 2,4-D that initiates the somatic embryogenesis remain to be elucidated.

MATERIAL AND METHODS

Ripe seeds of Norway spruce (*Picea abies* (L.) Karst.) were obtained from selected open pollinated trees in the Beskydy mountain area (680 m altitude). The seeds were surface sterilised by rinsing in 70% ethanol followed by 20 min. in 20% SAVO and rinsing five times in sterile distilled water. Half-strength LP medium (von Arnold, 1987) modified by Durzan

et al. (1994) was used in all experiments. The medium was supplemented with 20 μM BAP and 50 μM 2,4-D. For uptake, transport and metabolic studies ^{14}C-2,4-D (Sigma, labelled on carboxyl carbon, specific activity 1.147 GBq.mmol–l at 5.9 MBq.L^{-1}, which equals 5.1 μM 2,4-D) was added. Thus the total concentration of 2,4-D was 55.1 μM in the induction medium.

The intact embryos and/or their longitudinal halves were cultured in the dark at a temperature of 23 ± 2 °C in multi-well plates (24 wells, 1 ml of medium and 5 embryos per well). Each variant included 5 embryos and/or 10 halves with six repetitions.

Uptake of 2,4-D was studied on ZEs cultured on the medium for 1, 2, 4, 8, 16, 24, 32, 40, 48 and 72 hours. After this treatment period the embryos or their longitudinal halves were removed from wells and very briefly rinsed in distilled water to eliminate the remains of culture medium from their surfaces, and quickly surface dried with filter paper.

^{14}C-activities from ^{14}C-2,4-D (for simplification further only ^{14}C-activity is used) were detected after different treatment periods using liquid scintillation counting. After the drying of embryo parts to a constant weight (120 minutes in 80 °C), the tissues were combusted in a stream of oxygen (Kala and Peška, 1980). ^{14}C-activity was estimated using a PACKARD 2000 CA scintillation analyser. Data were corrected automatically for quenching. The ^{14}C-content was calculated from detected data.

Free and metabolised 2,4-D was detected after 1, 8, 16 and 48 hours in intact and/or in longitudinally bisected ZEs (treatment periods were chosen with regard to results from uptake experiments). Approximately 100 mg of a fresh mass of cultured explants was homogenised in 80% methanol with 100 mg·L^{-1} butylated hydroxytoluene and extracted for 1 hour on a shaker at 4 °C. The extract was centrifuged at 5000 x g for 15 min at 4 °C and purified on a C-18 column (Sep-Pak, Millipore USA). The purified extract was evaporated to dryness, dissolved in a small amount of methanol and applied to thin-layer chromatography (TLC) silica gel plate. The ratio of metabolised and non-metabolised 2,4-D was tentatively determined by means of TLC. In the first solvent system: benzene and acetic acid (75/12, v/v), free 2,4-D was separated from polar and non-polar metabolites. These metabolites were eluted and re-chromatographed in the solvent system: ethyl acetate, 2-butanone, formic acid and water (50/30/10/10, v/v/v/v) in which the metabolites could be separated.

RESULTS AND DISCUSSION

In Norway spruce ZEs the initial uptake of ^{14}C-2,4-D was detected in a preliminary experiment after one-minute treatment in liquid induction medium. The total level of ^{14}C-labelled compounds per mg of fresh weight during first three days was lower in bisected ZEs than in intact ZEs with a maximum at 16 hours (about 130 pmol) and a minimum at 40 hours on induction medium (about 7 pmol) (Fig. 1).

On the contrary the total levels of ^{14}C-labelled compounds per embryo that show the actual situation in single ZE were higher in bisected ZEs (two halves) than in intact ones until 16 hours when it reached a maximum. The level of ^{14}C-labelled compounds per mg of dry weight in bisected embryos was also higher (1.6x) than in intact ones. Centeno et al. (1999) reported similar increase of NAA uptake per mg of dry weight connected with wounding of petiolar tissues of *Actinidia*. Both auxins entered easily through the damaged tissues because of the absence of physical barriers such as cuticle in the cut areas, which also exposed a greater surface area for direct contact with the medium and therefore for NAA and 2,4-D diffusion. This diffusion was accompanied by water uptake, which modified the results of uptake per 1mg of fresh weight. So from this point of view the injury of ZEs resulted in a decrease of 2,4-D level. This was the reason for showing both results. Although the quantification of uptake per mg of dry weight should be more precise, uptake per mg of fresh weight seems to show more exact physiological status. Both methods are generally used in similar uptake experiments (Bronsema et al., 1996; Ceccarelli et al., 2000). In our experiments the level of ^{14}C-labelled compounds per embryo seemed to bee usable compromise.

After 16 hours the level of ^{14}C-labelled compounds per bisected embryo rapidly decreased. On the contrary the maximal level in intact ZEs was observed after 24 hours on induction medium and then started gradually decrease. Both maximal levels were similar with about 160 pmol of ^{14}C-labelled compounds (Fig. 2).

In fact in all our 2,4-D uptake studies we detected ^{14}C-activity not only in 2,4-D but also in its metabolites. In following experiment we concentrate on main groups of 2,4-D metabolites – non-polar and polar, as their presence could influence morphological responses. The biological activity is attributed to free 2,4-D and its non-polar metabolites. That is why the relative content of free ^{14}C-2,4-D, and its polar and non-polar metabolites were detected. Part of the ^{14}C-activity was deposited in an unidentified fraction.

Metabolism in all studied period was not an intensive process because the relative content of free ^{14}C-2,4-D was higher than the content of both types of metabolites together at all times regardless of whether in intact or bisected ZEs. In all variants the level of non-polar metabolites of ^{14}C-2,4-D was higher than the level of polar ones during the whole culture period.

The highest relative content of polar and non-polar metabolites was detected after 48 hours when it reached about 40% in intact ZEs and nearly 30% of the total ^{14}C-activity in bisected ones, respectively. The relative content of free ^{14}C-2,4-D was approximately the same until 16 hours on induction medium in intact ZEs while it was more variable in bisected ZEs (Tab. I).

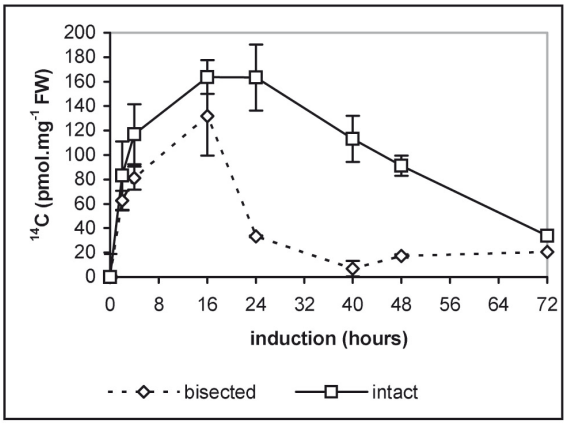
The average content of free ^{14}C-2,4-D and its metabolites in one intact and/or bisected ZE was calculated. After one hour on induction medium the intact and also bisected ZEs showed similar metabolic activity and content of free ^{14}C-2,4-D (Fig. 3A,B). On the contrary Bronsema et al. (1996) detected in immature maize ZEs the first metabolised 2,4-D from 2 hours onwards. Formation of polar metabolites (conjugates with amino acid and glucose) started in maize after 16 hours of culture as well as in intact ZEs of Norway spruce in our experiments (Fig. 3A). On the contrary their bisection resulted in earlier formation of polar metabolites even onward 8 hours (Fig. 3B). Our results suggested changes in ratio of metabolites and free 2,4-D caused by bisection. Relative amount of metabolites was lower in bisected ZEs till 16 hours (Fig. 3B). It was probably not the reason of lower metabolic activity, because the level of total metabolites were approximately the same in both variants, but the reason of the higher uptake of free ^{14}C-2,4-D from medium. The biggest difference in bisected ZEs was observed after 48 hour when the levels of free 2,4-D and metabolites were found similar to levels reached after 1 hour (Fig. 3B). In the same time the intact ZEs showed much higher levels with the relatively highest level of metabolites (Fig. 3A).

These differences might be connected with cytosolic acidification as a consequence of injury, which was reported by Hara et al. (2000) in tobacco plants. It could be one of the factors that affects metabolic pathways and uptake of 2,4-D in bisected ZEs. In our experiments the ZEs were injured artificially by bisection but they can also be injured even during normal isolation. This could explain different results in different laboratories.

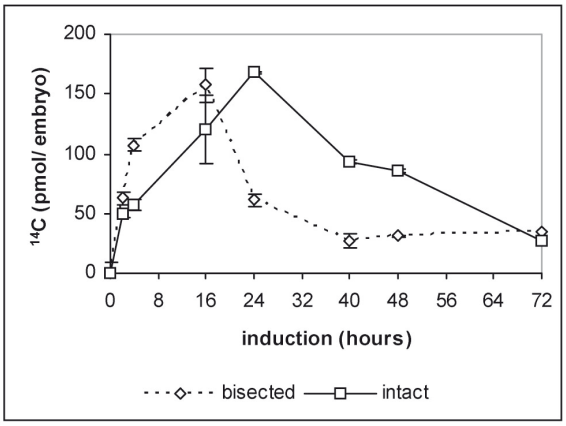
I: *Differences in level of 14 C-2,4-D and its metabolites between intact (A) and longitudinally bisected (B) zygotic embryos after various induction interval*

A	**metabolites (%)**			**free**	**undefined**
Hours	**polar**	**non-polar**	**total**	**^{14}C-2,4-D (%)**	**^{14}C (%)**
1	2.3	13.80	16.10	58.0	25.90
8	1.1	23.30	24.40	59.3	16.30
16	9.3	19.50	28.80	59.5	11.70
48	18.8	21.55	40.35	49.1	10.55

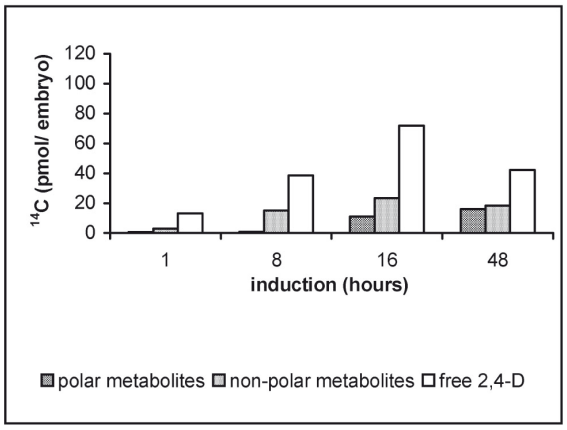
B	**metabolites (%)**			**free**	**undefined**
Hours	**polar**	**non-polar**	**total**	**^{14}C-2,4-D (%)**	**^{14}C (%)**
1	3.0	11.6	14.6	54.0	31.4
8	7.9	8.7	16.6	68.4	15.0
16	6.6	18.3	24.9	60.5	14.6
48	2.6	27.3	29.9	42.9	27.2



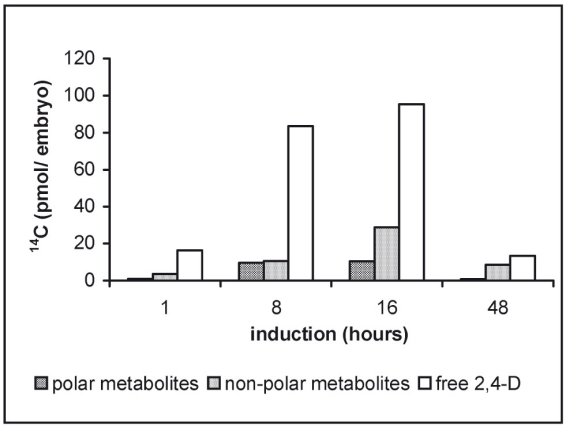
1: *Effect of induction interval on total content of ^{14}C-labelled compounds per mg of fresh weight in intact and bisected zygotic embryos. Confidence intervals are indicated by vertical bars when larger than the symbols in all figures (P<0,05)*



2: *Effect of induction interval on average content of ^{14}C-labelled compounds per embryo in intact and longitudinally bisected zygotic embryos*



3A: *Effect of induction interval on metabolic activity of intact zygotic embryos*



3B: *Effect of induction interval on metabolic activity of longitudinally bisected zygotic embryos (The total average levels of ^{14}C-labelled compounds per embryo were detected on the basis of the previous experiment (see figure 2))*

SUMMARY

Uptake and metabolism of 2,4-dichlorfenoxyacetic acid (2,4-D) into zygotic embryo of Norway spruce during induction of somatic embryogenesis was the main topic of this study. The wounding of zygotic embryo has been found as the second factor, which influenced frequency of developed embryogenic cultures. The longitudinally bisection of embryos resulted in lower level of ^{14}C-labeled compound detected per 1 mg of fresh weight because of higher water uptake. On the contrary, the level of ^{14}C-labeled compound detected per 1 mg of dry weight and/or per one embryo was higher in bisected embryos till 16 hours in their culture, then rapidly decreased. Intact embryos reached maximum ^{14}C-activity after 24 hours in culture and then gradually decreased. Both cultures reached similar maximal and minimal

levels. The further experiment was focused on ability of intact and bisected embryos to metabolise ^{14}C-2,4-D. Bisection of embryos resulted in modification of metabolic activity. Earlier production of inactive polar metabolites was found even after 8 hours in their culture, while intact embryos produced them after 24 hours. Bisection resulted in faster release of ^{14}C-labeled compounds back into medium, the levels after 48 hours decreased on the approximately same low level as after first hour in culture. In our experiments the ZEs were injured artificially by bisection but they can also be injured even during normal isolation. This could explain different results in different laboratories.

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SOUHRN

Příjem a metabolizmus 2,4-dichlorophenoxyoctové kyseliny během indukce somatické embryogeneze smrku (*Picea abies* (L.) Karst.)

Předložená práce se zabývá studiem příjmu 2,4-dichlorfenoxyoctové kyseliny do zygotických embryí smrku během indukce somatické embryogeneze. Tento růstový regulátor jev této metodě klíčovým faktorem. Dalším faktorem, který může ovlivnit frekvenci získaných embryogenních kultur, je podélné dělení embryí. Protože tyto dva faktory spolu úzce souvisejí, bylo cílem této naší práce zjistit rozdíly v příjmu 2,4-D do intaktních a podélně dělených embryí pomocí ^{14}C-2,4-D. Při přepočtu na 1mg čerstvé hmotnosti se příjem do dělených embryí jevil jako nižší v důsledku rychlého příjmu vody. Pro srovnání byly zjištěné aktivity přepočítány na jedno embryo a bylo zjištěno, že do 16 hodin byl příjem ^{14}C-2,4-D rychlejší u dělených embryí. To bylo potvrzeno i přepočtem na 1 mg sušiny. Dělená zygotická embrya přijala po 16 hod 1,6x více ^{14}C-2,4-D než intaktní. Intaktní embrya dosáhla maximální hladiny až po 24 hodinách. Po dosažení této hodnoty hladina ^{14}C-2,4-D pozvolna klesala, naproti tomu u dělených embryí klesala prudce již od 16 dodiny indukce. Maxima i minima měla přibližně stejné hodnoty u obou variant. Protože při indukci somatické embryogeneze hraje roli kromě příjmu 2,4-D i schopnost pletiva tento syntetický auxin metabolizovat, byly v této práci porovnány i rozdíly v obsahu metabolitů v dělených a intaktních embryích. Bylo zjištěno, že poraněním embryí došlo k rychlejší tvorbě fyziologicky neaktivních polárních metabolitů (už po 8 hodinách), kdežto u intaktních embryí byly tyto metabolity detekovány až po 16 hodinách. Relativní podíl volné a metabolizované ^{14}C-2,4-D byl do 16 hod vyšší u dělených embryí, celkové aktivity metabolizované ^{14}C-2,4-D se ale výrazně nelišily. Po 48 hod se projevilo výrazné snížení hladiny volné i metabolizované ^{14}C-2,4-D u dělených embryí na úroveň stejnou jako po 1 hod indukce. V našich experimentech byla embrya poškozena záměrně a byly zjištěny významné rozdíly v příjmu i metabolizmu 2,4-D. K poškození embryí může docházet i neúmyslně během preparace, což může být jednou z příčin odlišných výsledků v různých laboratořích.

2,4-dichlorophenoxyoctová kyselina, smrk, somatická embryogeneze

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