MYROSINASE ACTIVITY IN DIFFERENT ORGANS OF *BRASSICA NAPUS* L.

Atle Bones
Department of Botany, University of Trondheim,
N-7055 Trondheim, Norway.

Abstract

Myrosinase activity in extracts from seeds, seedlings, regenerated shoots and plants and mature plants of *Brassica napus* L. was determined by the rate of glucose formation from glucosinolate hydrolysis. Calli with shoots and regenerated plants were obtained from protoplasts derived from hypocotyls.

The crude enzyme preparations from different organs of seedlings were prepared from seedlings germinated in the light. Hypocotyls showed the highest myrosinase activity with a maximum of 0.520 umol· min-^{1} mg-^{1} In cotyledons a nearly constant enzyme activity of approximately 0.13 umol·min^{-1}mg^{-1}was determined over the first 6 days, followed by a gradual decrease to 0.013 umol· min^{-1}· mg^{-1} at the 20th day. Roots showed the highest decrease in enzyme activity over the investigated period, changing from ca. 0.40 to 0.02 umol·min^{-1}·mg^{-1}Leaves, inflorescence and stems from flowering regenerated or "normal " plants contained usually less than 0.005 umol.min^{-1}·mg^{-1}.

Keywords

Beta-thioglucosidase (myrosinase), plant regeneration, protoplast, dilated cisternae.

Introduction

The agricultural importance of the Brassicaceae is related to the occurence of glucosinolates and their hydrolytic enzymes, myrosinase (beta-thioglucosidase, E.C.3.2.3.1). They are responsible for the hydrolysis of glucosinolates to isothiocyanates, glucose and sulphate in neutral solution (see e.g. Wilkinson *et al.*, 1984). It has been reported that myrosinase occurs in a special type of idioblast, named myrosin cells. This is based on evidence from unspecific protein reagents (Bones and Iversen, 1985).Several articles describe the occurence and distribution of myrosin cells in Brassicaceae species (Iversen *et al.*, 1979; Rest and Vaughan, 1972; Werker and Vaughan, 1974, 1976; Jørgensen, 1981; Bones and Iversen, 1985).

Detailed investigations have been made of myrosinases extracted from seeds of *Brassica napus* L. and the enzymes have been purified and characterized (Lönnerdal and Janson, 1973; Kozlowska *et al.*, 1983). There have been few reports with systematic analysis of the variation in myrosinase activity in different development stages and organs. Pihakaski and Pihakaski (1978) followed the distribution of myrosinase in various morphological parts of *Sinapis alba* L. and Iversen and Baggerud (1980) and Wilkinson *et al.* (1984) examined the activity in some organs of several species.

The aim of the present study was to follow the variations in myrosinase activity in plant tissue from *Brassica napus* L. at various differentiation and developmental stages. For this purpose it was decided to use seedlings, flowering plants and regenerated shoots and plants from protoplasts. Attempts have also been made to find myrosin cells in undifferentiated tissue (callus).

Material and methods

Seedlings.

Seeds of *Brassica napus* L. cv. Niklas (rape) were surface sterililized in 15% Klorin for 45 min, washed four times in distilled water and germinated in petri dishes on 1% agar (Difco) with 0.2% sucrose and 3.4 mM $CaCl_2 \cdot 2H_2O$. The photoperiod was 16 h light (fluorescent ca 2500 lux). Seeds were kindly provided by Svalöf AB, Sweden.

Protoplast isolation.

Protoplasts were isolated from 8 days old seedlings after the method described by Glimelius (1984). Hypocotyls were preplasmolyzed 45 min in a solution containing 0.3 M sorbitol and 0.05 M $CaCl_2 \cdot 2H_2O$. After an incubation period of 12-14 h in culture medium A (Kao and Michayluk, 1981) supplied with 1% Cellulysin, 0.5% Macerozyme, 1.0 $mg \cdot l^{-1}$ 2,4-D, 0.5 $mg \cdot l^{-1}$ BAP, 0.1 $mg \cdot l^{-1}$ NAA and 0.4 M sucrose, the mixture was poured through a nylon sieve (63 um) and diluted with a salt solution (Banks and Evans, 1976) with 0.47 M sucrose. The protoplasts were collected by centrifugation and washed in W5-solution (Menczel et al.,1981) and culture medium. Protoplasts were cultured at a density of $2\text{-}4 \cdot 10^4 \cdot ml^{-1}$.

Plant regeneration.

Microcalli obtained from the protoplast cultures were transferred to culture medium A supplied with 0.05 M sucrose, various concentrations of zeatin and 0.11 $mg \cdot l^{-1}$ IAA. Callus with regenerated shoots were planted in pots and grown under green-house conditions.

Enzyme assay.

Tissue was crushed in a mortar with sand and 0.01 M imidazole-HCl buffer (pH 6.2). The homogenate was centrifuged at 12 000 g for 20 min at 4°C and the supernatant dialyzed against the same buffer. This crude extract was tested using GOD-Perid-reagent to determine the amount of glucose liberated from hydrolysis of sinigrin. The activity is given as umol glucose liberated per minute per mg protein. The protein content was measured by the method of Lowry *et al.* (1951) using bovine serum albumin standards.

Microscopy.

Tissue segments were fixed in 3% glutaraldehyde for 2 h at 21°C, washed in 0.1 M phosphate buffer (pH 7.2) and post-fixed for 2 h in 2% OsO_4 in the same buffer. Dehydration was carried out in ethanol of increasing concentrations and the tissue was embedded in LX 112 . Semithin and ultrathin sections were cut on a LKB-Ultrotome IV fitted with diamond knife. The sections were stained for 20 min in 1% uranyl acetate followed by lead citrate for 10-15 min and examined in a JEOL 1200-EX electron microscope.

Results

Regeneration of plants.

Protoplasts were readily isolated from the source investigated (Figure 1), and the microcalli obtained were capable of shoot regeneration on media supplemented with several hormone combinations (Table 1). Most reproducible regeneration frequencies were obtained when zeatin was used in combination with IAA in the regeneration medium (Figure 2).

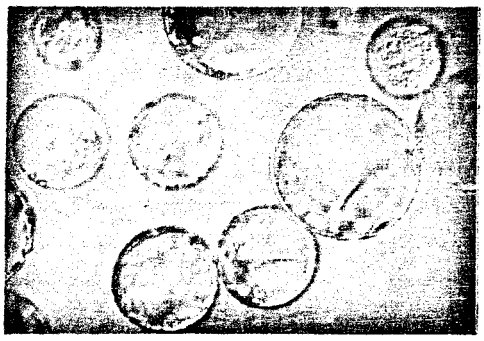

Figure 1. Freshly isolated protoplasts from 8 days old hypocotyls of rape.

Medium	Zeatin (mg·l^{-1})	6-BAP (mg·l^{-1})	IAA (mg·l^{-1})	NAA (mg·l^{-1})	Shoots	Roots
A		10			-	+
A		25			+	+
A		10		0.10	-	+
A		25		0.05	-	+
A		25		0.10	-	+
B5		10			+	+
B5		20			+	+
B5		25			+	+
B5		10		0.10	-	+
B5		20		0.10	-	+
B5		25		0.10	-	+
A	1		0.11		+	+
A	2		0.11		+	+
A	3		0.11		+	+
A	4		0.11		+	+
A	5		0.11		+	+
A	6		0.11		+	+
A	8		0.11		+	+
A	10		0.11		+	+

Table 1. Evaluation of the differentiation response of some hormone combinations and media. (+ and - indicate positive and negative response respectively)

Myrosinase activity in seedlings

The myrosinase activity in seedlings of *Brassica napus* L. varies from organ to organ and with the age of the plants (Figure 3). Specific myrosinase activity determined using the crude dialysed enzyme preparations, was always higher in hypocotyls than in roots and cotyledons at the same stage of development. The enzyme activity in hypocotyls showed maximum value two days after germination, followed by a nearly hyperbolic decrease over the next 18th days Figure 3).

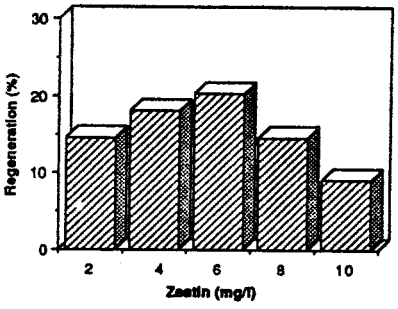

Figure 2. The percentage of protoplast derived calli of rape producing shoots in medium A with 0.11 mg · l^{-1} IAA and various concentrations of zeatin.

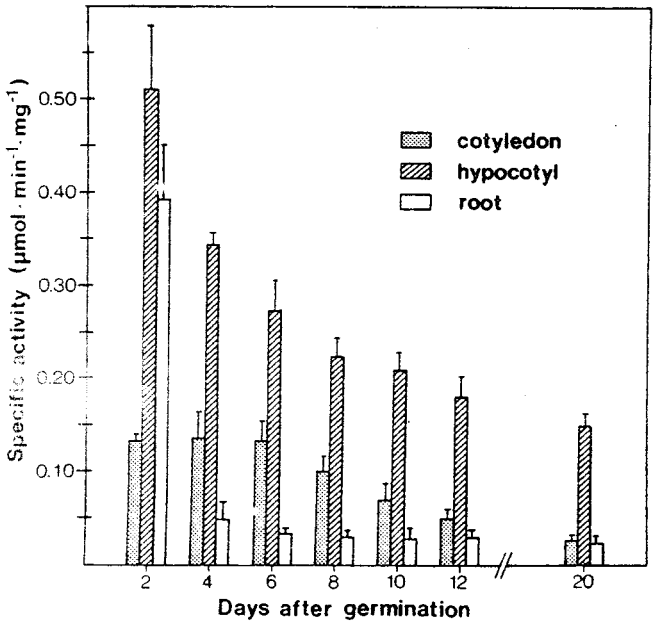

Figure 3. Myrosinase activity in cotyledons, hypocotyls and roots of rape during development of seedlings (mean ± standard deviation).

In cotyledons a nearly constant enzyme activity of approximately 0.13 umol· min^{-1}· mg^{-1} was determined over the first six days, followed by a gradual decrease to 0.013 umol· min^{-1}· mg^{-1} at the 20th day (Figure 3). A marked fall in myrosinase activity in roots of rape seedlings was observed during the first period of germination, where the activity decreased from 0.39 to 0.05 umol· min^{-1}· mg^{-1}.

Myrosinase activity in calli with shoots.

For routine analysis, shoot formation on calli derived from protoplasts was obtained after *in vitro* culture on A-medium supplemented with 8 mg· l^{-1} zeatin and 0.11 mg· l^{-1} IAA. A

comparison of the myrosinase activity in differentiated and undifferentiated calli are shown in Table 2.

Source	Myrosinase activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
Differentiating callus	0.0112 ± 0.0057
Undifferentiating callus	0.0020 ± 0.0009

Table 2. Myrosinase activity in differentiated and undifferentiated calli.

Myrosinase activity in flowering plants.

Even low, myrosinase activity was detected in all samples from different organs of flowering rape plants. Leaves, stems and inflorescence contained normally less than 0.005 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (Table 3). Young leaves near the apical bud usually showed more activity than older leaves. In contrast specific myrosinase activity in stems was highest in the oldest parts. No significant difference were found between plants derived from seeds and plants regenerated from protoplasts (Table 3).

Organ	A ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	B ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
Inflorescence	0.0040 ± 0.0022	
Upper leaves	0.0033 ± 0.0012	0.0029 ± 0.0013
Lower leaves	0.0020 ± 0.0004	0.0015 ± 0.0006
Upper stem	0.0017 ± 0.0006	0.0016 ± 0.0006
Roots	0.0223 ± 0.0063	0.0243 ± 0.0072

Table 3. Specific myrosinase activity in organs of flowering rape plants derived from seeds (A) or regenerated from protoplasts (B). Values are expressed as mean ± standard deviation.

Microscopy.

The ultrastructure of rape calli are shown in Figure 4. So far, no myrosin cells have been found in calli cultures of rape. However, dilated cisternae of the endoplasmic reticulum (DC) which has been associated with myrosinase, appeared in all calli examined (Figure 4B). The DC usually contained a homogeneous granular material, but DC with tubular-like structures has also been observed.

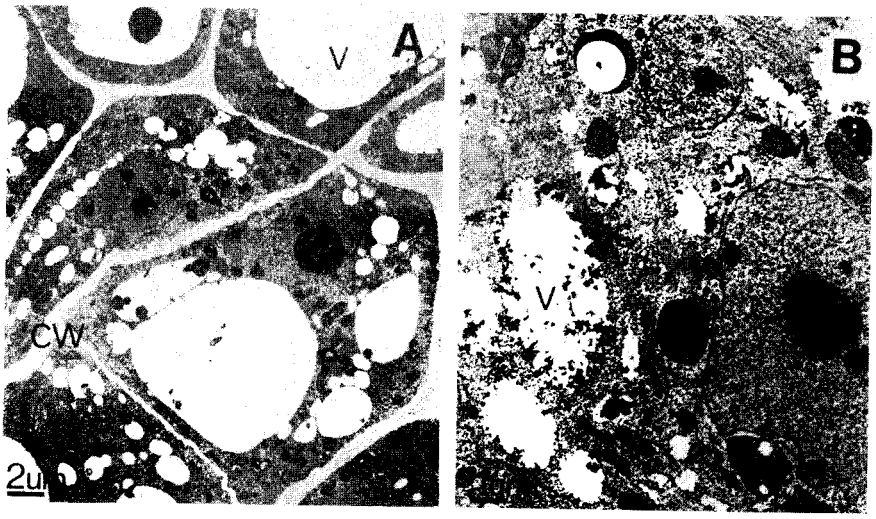

Figure 4. Regenerated calli from protoplasts of *Brassica napus* L. (A) Microcallus grown for 14 days in A-medium and (B) microcallus grown 28 days . Abbrivations: cell wall (CW), vacuole (V), dilated cisternae of the endoplasmic reticulm (DC), mitochondrion (M), plastid (P).

Discussion

The results described here show that hypocotyls contain the highest myrosinase activity of the seedling organs. Similar results were reported by Pihakaski and Pihakaski (1978) who found the highest specific activity in hypocotyls and the least activity in primary roots in three days old seedlings of *Sinapis alba* L. A denser staining and quicker development of myrosinase bands with samples from hypocotyls compared to samples from cotyledons, was observed by Phelan and Vaughan (1980) in a study of the myrosinase isoenzyme pattern in *Sinapis alba* L. They also observed that the pattern of bands in cotyledons remained the same as in seeds, but with a slightly clearer definition the first 72 h after germination. In the present study myrosinase activity in cotyledons was stable for the first 6 days after germination (Figure 3).

In general myrosinase activity in seedlings decreases with ageing of the plants (see e.g. Iversen and Baggerud, 1980). Phelan *et al.* (1984) were unable to detect myrosinase activity in cotyledons of *Raphanus sativus* L. after the plant had developed the first pair of permanent leaves. The latter observation is not confirmed by our results, where enzyme activity was detected in all plant samples tested.

The myrosinase activity in calli with shoots was much higher than in control calli without shoots (Table 2). This may together with the high activity in seedlings indicate that myrosinase is involved in growth promotion as suggested by Pihakaski and Pihakaski (1978). An increased enzymatic activity in differentiating cultures as compared to undifferentiated cultures has however, also been reported by Malpathah and David (1986). They measured a three fold increase in phenylalanine ammonia-lyase activity in *Allium sativum* L. calli with shoots. It should also be stressed that the myrosinase activity in differentiating calli showed considerable more variation than undifferentiating calli.

At the flowering stage, regenerated plants showed only minor differences in enzyme activity as compared to normal plants, and the regenerated plants had the same distribution of myrosinase activity as normal plants.

It has been discussed whether or not there is a correlation between the occurence of myrosin cells, dilated cisternae of the endoplamic reticulum and /or myrosinase activity. Phelan and Vaughan (1980) and Phelan *et al.* (1984) claimed that there is a correlation between myrosin cells and myrosinase in sedlings of *Sinapis alba* L. and *Raphanus sativus* L., except for primary roots. The latter is supported by Iversen et al. (1979) who determined myrosinase activity but not myrosin cells in 48 h old roots.

Dilated cisternae of the endoplasmic reticulum have been reported to occur in intact plants of several species (see Jørgensen , 1981), in freshly isolated protoplasts (Iversen *et al.*, 1983), in leaf and root explant callus of *Armoracia rusticana* (Gailhofer *et al.*, 1979) and are here reported in protoplast derived calli. The occurence of DC and myrosinase activity, together with the abscence of myrosin cells in rape calli indicate that myrosin cells do not seem to be a prerequisite for enzymatic activity.

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