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[1]University of Hamburg, Ecology and Biology of Useful Plants, Biocenter Klein Flottbek, Hamburg, Germany
[2] University of Joensuu, Research and Teaching Laboratory of Applied Botany, Faculty of Biosciences, Joensuu, Finland

Polyphenol oxidase (PPO, catecholase) activity during germination and early seedling growth of Cicer milkvetch (*Astragalus cicer* L.)

Rasim Sahbaz[1], Reinhard Lieberei[1], Tadeusz Aniszewski[2]

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Summary

Mature seeds of the legume *Astragalus cicer* L. form a homogeneous seed pool in size and weight. They occur either brown coloured or yellow coloured. They reveal an expressed dormancy but they germinate readily after mechanical scarification. Time span of germination and early seedling development under defined conditions were followed and compared to the activity and potential activation of polyphenol oxidase (PPO, catecholase). The yellow seeds developed without difficulties, but on the brown coloured seeds in the course of germination hyphal growth was visible which was linked with the death of the seedlings. Quantitative experiments with the yellow seeds revealed that scarified seeds formed more than 98 % of seedlings within seven days. There was no difference of germination rate between dark and daylight incubations. The polyphenol oxidase of the seeds occurs in two forms, a preformed testa localized enzyme and an induced PPO activity of the seedling. The initially high activity of the testa related PPO (1.32 µM O_2/h x g fresh weight) decreases during the first days after germination, the activity of the seedling related PPO becomes detectable four to five days after onset of germination. After seven days the activity in roots is about 3.6 µM O_2/h x g fresh weight. The cotyledons reveal 2.3 and the hypocotyl 1.9 µM O_2/h x g fresh weight, respectively.
There is an indication that autoinhibitory components interfere with the germination process. The importance of the polyphenol oxidase as a component of the plants' protection system during germination is discussed.

Introduction

Polyphenol oxidases (PPOs) are almost ubiquitous among all group of organism and they have been related to defence mechanism against pathogens and herbivores but much is still unclear about their biological function. They are widely occurring enzymes among plant kingdom. They catalyze browning reactions in injured tissues and are of special commercial importance because of their induced browning and quality impairment of fruits, vegetables and fodder plants. The browning reactions occur due to the oxidation of phenolic constituents after damage of cells of intact plant materials. Due to their general occurrence it is assumed, that they may play a role in biochemical protection of sensitive plant developmental stages against microbes (MAYER, 1987; ANISZEWSKI et al., 2008). Polyphenol oxidases in general are various enzymes, which can be arranged due to their reaction mechanisms and substrate specificity into three different groups: Catechol oxidases that oxidise o-diphenols to o-diquinones (EC 1.10.3.2), laccases that oxidise p-diphenols to p-diquinones (EC 1.10.3.1), and tyrosinases (in animals) or cresolases (in plants and microbes) that are PPO-type enzymes with an additional function for hydroxylation of monophenols to o-diphenols (EC 1.14.18.1) (MAYER, 1987; ANISZEWSKI et al., 2008). In intact plant tissue the polyphenol oxidases, respectively the catecholases, are widely found to be located in the thylakoid membranes of the plastids, which was firstly described by ARNON (1949). Interestingly, new molecular studies on the „model plant" *Arabidopsis thaliana* revealed, that in the genome of this member of the *Brassicaceae* no genes coding for catecholases have been found, but, instead, genes coding for the laccase enzyme. There is no evidence for the occurrence of active laccase in members of the Fabaceae.

Astragalus cicer is a herbaceous legume with hollow stems, pubescent, broad, membranous leaflets on pinnately divided leaves. The root system is vigorously creeping. The inflorescence is from 10 to 30 cm white flowered raceme and the pods are hairy and short. Cicer milkvetch is a long lived perennial with potential to serve as a forage plant.

In biochemical ecology many plants reveal various protection mechanism and especially legume plants often contain nitrogen containing toxic compounds like alkaloids, cyanogenic glycosides or non proteinogenic amino acids. In many legumes the testa is characterized by strong cellular layers which give rise to dormancy (HALL et al., 1989; ANISZEWSKI et al., 2006). Though the occurrence of some enzymes in the testa has recently been described (ANISZEWSKI, 2005, 2006), up to now no studies have been reported about the occurrence of defense or protection related enzymes in these cell layers in *A. cicer*. Reports about occurrence and activity of PPO in this plant so far do not exist. Therefore the objective of the present study was to study if PPO in Cicer milkvetch may reveal a possible defense function in the sensitive early phase of germination and seedling development.

Material and methods

Germination conditions

Cicer milkvetch is a long lived perennial which grows in scattered localities from southwest Asia, Siberia to Western Europe. It grows naturally on margins of paths, verges, fields, and along railway tracks (TOWNSEND, 1975; HULINA, 1996; FEDER, 1998). Cicer milkvetch is adapted to a wide range of soil types. It produces high amounts of seeds with expressed low germination rates of less than two percent. Cicer milkvetch seeds are very hard and small (Fig. 1 and Tab. 1a). They occur in two different colours, brown and yellow. The yellow seeds developed without difficulties, but on the brown coloured seeds in the course of preliminary experiments hyphal growth was visible which caused the death of the seedlings by fungal attack.
Freeze-thaw treatments and mechanical scarification reduce hard seed character and increase germination and emergence. Mechanical scarification can be done without damaging seeds (MALEK et al., 1998).
Both the light and dark cultured seedlings were positioned in equal distances in petri dishes on moderately moisturized filter paper (3 ml tap water per petri dish of 10 cm diameter). 10 seeds per dish for comparative experiments for light and for dark culture were maintained at 21 C. Plantlets for experiments to measure PPO activity were raised using glas dishes of Ø 15 cm and 3 cm height under day light culture at 21 C.

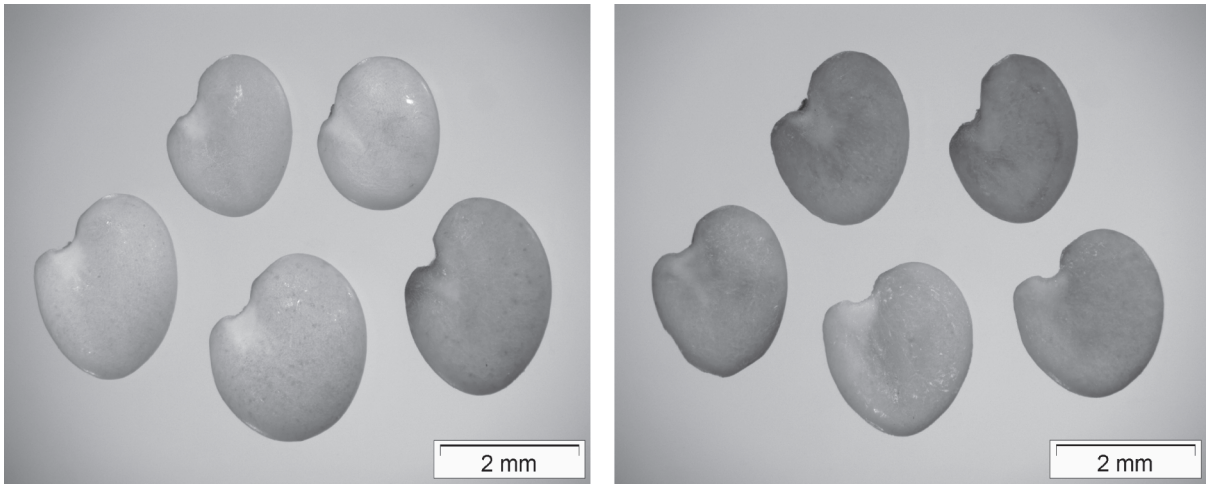

Fig. 1: Yellowish seeds (left) and brown seeds (right) of *A. cicer*, pictures taken with Olympus SZX9 mi and Soft imaging system AnalySIS®. The colour variation within the groups is shown in Fig. 1.

Autoinhibition, germination of scarified and non-scarified seeds

In order to check if water soluble substances are exuded from germinating seeds or if gaseous compounds are liberated during the early germination process, 20 scarified seeds were placed in outside part of Conway diffusion chambers together with 3 ml of „germination solution" and 20 non-scarified seeds in centre case of the Conway vessel. The germination solution was taken from 3 days old scarified seed culture (see Fig. 3). For the controls tap water was used with 5 replicates.

PPO (catecholase) estimation

1 to 7 days old seedlings and isolated seed shells were used for PPO activity measurement. After separation of the testa from the repective seedling the plant material was homogenized with mortar and pestle in cold phosphate buffer composed of KH_2PO_4 and Na_2HPO_4, according to Sörensen (67mM) at pH 6.4 using 1 ml per g plant material. The homogenate was centrifuged for 10 min at 13.000 x g, 4 °C (Biofuge fresco Heraeus Christ).

The PPO activity was determined polarographically at 25°C with a Yellow Springs Instruments oxygen electrode 5331 (YSI, Yellow Springs, Ohio, USA). The air saturated reaction mixture contained 2.4 ml of 67 mM phosphate buffer pH 6.4, 0.5 ml of supernatant and 0.1 ml 4-methyl catechol at a final concentration of 7.5 mM in 67 mM phosphate buffer. This substrate is valid to detect the catecholase activity. The occurrence of cresolase and of laccase was excluded in preliminary tests.

We followed the procedure described by LIEBEREI et al. (1981). For the test of potential activation capacity or latent PPO, respectively, the non-ionic detergent Triton X-100 was added to the reaction assay to a final concentration of 1.0 %. The PPO activity was measured after addition of detergent in 10, 20, 60 and 120 min. Triton X-100 does not enhance the enzyme activity in *A. cicer* (data not shown). In order to check whether the oxygen consumption during the reaction was really due to PPO a KCN solution in phosphate buffer was added to the reaction mixture to a final cyanide concentration of 1 mM. At this cyanide concentration the PPO based oxygen consumption is completely inhibited.

Determination of seeds size

The weight and the size of 20 mature randomly selected yellow seeds were measured. The seeds were weighed by Libra „Sartorius micro" (Company Sartorius GmbH Göttingen Germany). For size estimation photos were taken on millimetre paper using Canon Power shot G5 combined with a Binocular Zeiss (Germany). For calibration the sizes of the seeds were evaluated by Soft imaging system AnalySIS®.

Results

Germination of scarified and non scarified seeds

The seeds of *A. cicer* form a morphologically relatively homogeneous group (Tab. 1a). They are highly dormant (Tab. 1b). The testa-related dormancy can be overcome by mechanic scarification. All scarified seeds took up water very quickly and liberated yellow-brown compounds into the medium within two to three hours of germination, whereas non-scarified seeds did not show any changes. This allows the assumption that the coloured compounds were not extracted from the testa surface but were liberated from the opened scarified seed. The germination of light and dark cultured seeds at room temperature did not lead to significant differences. After one day of germination of scarified seeds the testa could be separated from the germinated seeds and could be prepared for measurement of PPO activity. After two days in light culture 44.2 % of scarified seeds were germinated (Fig. 2). The corresponding germination fluid was brown coloured whereas the solution in the water-control was not changed.

The main progression in germination was from the second to the third day, where over 80 % of seeds could be classified as germinated. On the fourth day about 95 % of all seeds were germinated and finally 98,9 % germinated seeds were reached. In the group of non scarified seeds no germination at all could be registered and no brown discolouration of the germination medium occurred.

In summary the seeds can germinate perfectly when the testa is broken and water uptake is allowed, further on some substances are liberated during the phase and in addition to this it can be stated that the germination is not light dependent.

Modification of Germination

Germination of scarified seeds was carried out in tap water (control) or in germination medium taken from germinating scarified seeds after three days (Fig. 3). All seeds incubated in tap water germinated after three days whereas the seeds incubated in three days old germination medium needed one day longer for reaching the same status of complete germination. Non scarified seeds did not germinate

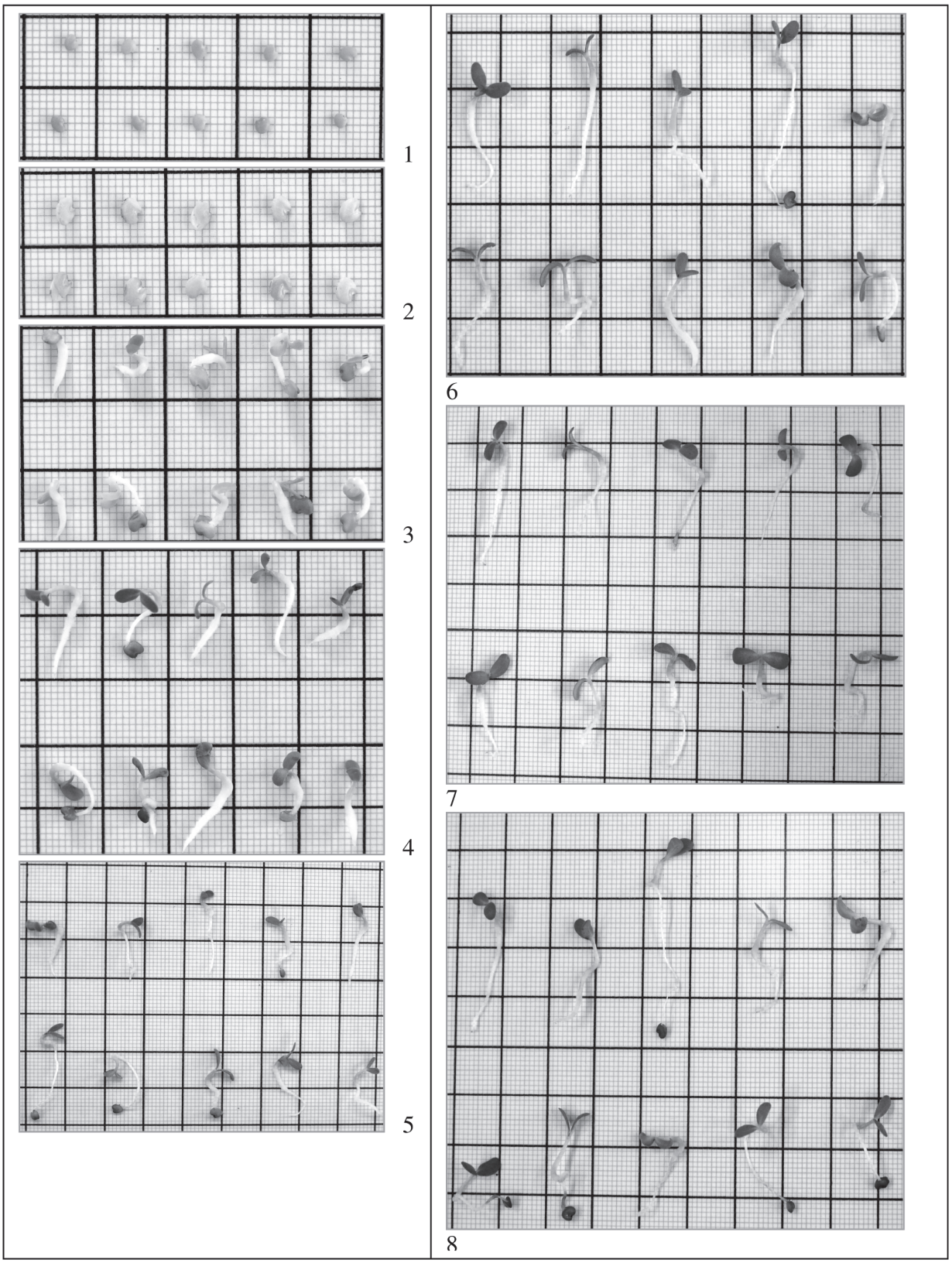

Fig. 2: Germination of *A. cicer* seeds in light conditions in the course of eight days.

at all, irrespective the time. Even after two weeks of incubation no germination did occur (data not shown).

Seedling development was better when the germination medium was substituted by fresh tap water. This was controlled over three days time with daily change of the medium. Root darkening was used as a qualitative indicator for changes in the seedling caused by factors of the germination fluid. It must be assumed that these factors were compounds produced in the germination process.

Fig. 3: Germination of scarified and non-scarified seeds after 4 days: left Conway diffusion chamber contained tap water, right Conway vessel contained germination solution from 3 days culture. The seeds in the inner compartment of both Conway vessels were non-scarified and the seeds in the outside compartment were scarified.

Tab. 1: Seed characters and germination rate of *Astragalus cicer* during early vegetative development

1a) seed characters

Weight [mg]	Length [mm]	Broadness [mm]	Thickness [mm]
4.22 ± 1.55	2.57 ± 0.14	2.08 ± 0.16	0.93 ± 0.09

1b) germination process

	Weight of testa		germination rate of scarified seeds		germination rate of non-scarified seeds		Weight of seedlings	
	Light culture	dark culture	light culture	dark culture	light culture	dark culture	light culture	dark culture
age of culture	[mg]	[mg]	[%]	[%]	[%]	[%]	[mg]	[mg]
1. day	6.46± 0.33	6.09± 0.38	100 % swollen	100 % swollen	non-swollen	non-swollen	10.76±1.10	11.88±0.78
2. day	5.17± 0.30	4.99± 0.35	44.22	42.40	0	0	27.50±1.22	25.54±1.12
3. day			83.55	82.80	0	0	29.14±2.17	27.35±1.54
4. day			96.44	95.20	0	0	36.03±2.04	32.10±2,40
5. day			98.66	97.60	0	0	39.69±1.37	35.06±1.59
7. day			98.88	98.40	0	0	43.08±1,60	38.62± 1,87
	n = 20	n = 20	n = 700	n = 700	n = 250	n = 250	n = 20	n = 20

PPO (catecholase) activity

Relation to plant development

Germinating seeds and developing seedlings reveal a good detectable PPO activity. In seeds one day after onset of germination the activity of PPO per fresh weight was highest (Fig. 4). The activity was comparable irrespective the absence or presence of light. In both lines, the PPO activity of both cultures decreased rapidly and reached the lowest value at the third day of culture. The enzyme activity decreased stronger in seedlings in the dark culture than in the light culture. On the fourth day of germination, roughly characterized by the visible formation of the seedling organs, PPO activity of both culture lines increased again.

Relation to seedling organs

In order to get information about PPO activity present in seed and seed shell and about the development of the activity in the course of seed germination, seedling formation, the enzyme activity was followed as detailed as possible (Fig. 5).

PPO activity was measured in the testa after the first and the second day of incubation. The activity remained unchanged over the two days. The testa was taken out of the incubation dishes after two days, because they were all separated from the seedling and did not contribute anymore to the seedling physiology and the seedling development. The swollen seed after the first day of incubation and the young seedling characterized by a small protrusion of the radicula were analysed without the testa. PPO activity in one day old swollen seeds was two times higher than the testa activity, but this activity decreased from the second day of culture to the third day of culture. This reduction of activity is not due to enzyme inactivation but must be related to the high water uptake (see Tab. 1b), because the enzyme activity is related to the fresh weight of the organs. From the third to the seventh day the seedling PPO activity increased again. The PPO activity was present in considerable height in all seedling organs (cotyledons, hypocotyls and roots) and was heighest in the roots. In

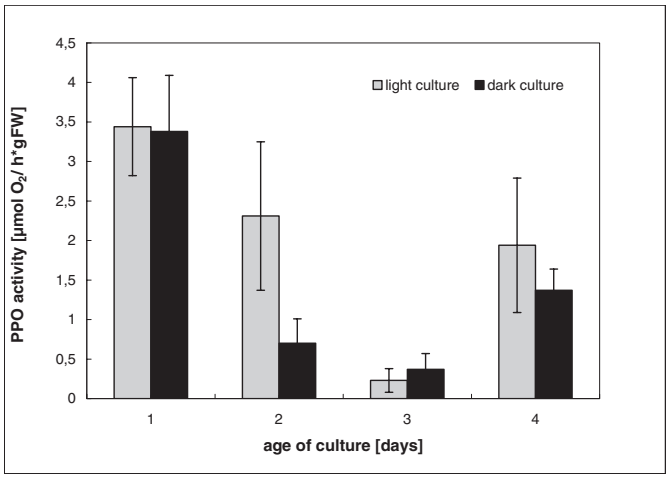

Fig. 4: PPO activity of complete seedlings in light and dark germination. (n=7). The enzyme activity is expressed as oxygen consumption per time and per fresh weight. The fresh weight of the seed or seedling, respectively, is varying due to the swelling process of the storage protein containing seed tissues. The water uptake per day is given in Tab. 1b in order to enable the comparative recalculation of the reference data.

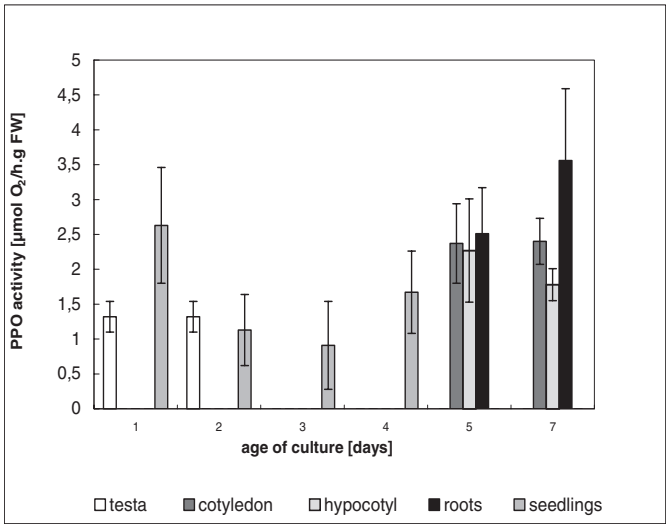

Fig. 5: PPO activity in testa, seedlings, and seedling organs of seeds cultured under daylight conditions.
For reference data of the activity per fresh weight the water uptake during swelling has to be taken into account, for explanation see legend of Fig. 4 (n=5 testa, n=7 other plant organs).

an activation experiment using Triton X-100 (1 %) (see also Material and methods: PPO-estimation) no activity changes were seen. This underlines the view that in this developmental stage no latent, membrane- or thylakoid-associated PPO is present.

Discussion

Effect of mechanical scarification on germination

Seeds of the potential forage legume *A. cicer* are dormant and do not germinate as needed for agricultural use. For laboratory tests a simple mechanical scarification test was applied to enable germination studies and to achieve rapid and effective germination.
The germination ability of seeds of some plant species, typically legumes, may be affected by the high rate of hard seed coats that inhibit water uptake (BASKIN and BASKIN, 1998). To improve germination and seedling emergence, seeds are either scarified mechanically (ACHARYA et al., 1999), by a treatment involving several freeze-thaw (-80°C/+20°C) cycles (HALL et al., 1998), chemically by immersion in sulphuric acid (TOMER and MAGUIRE, 1989; RUIZ and DEVESA, 1998), or by heat treatment (VALBUENA and TARREGA, 1998). The hard seed coats of *A. cicer* inhibit the absorption of water and prevent uniform germination as well as seedling growth. It was decided to apply the scarification method, as this method had been applied successfully to legume seeds of the same size and a comparable mechanical resistance (*Baptisia tinctoria*: VOSS et al., 1994).
The physiological state of seed material can play an effective role in the germination process. Therefore for uniform studies only yellow coloured mature seeds were used. These seeds revealed germination rate of more than 98 % after seven days of incubation. Brown seeds, also found in the seed pool were not used for the experiments.
These results indicate that the hard seeds coats are basic factor for germination inhibition of *A. cicer* seeds. Of course it is possible that other factors like incubation conditions, plant species or seed substances can influence the germination process but the results reveal significantly that the hardseededness of *A. cicer* seeds caused by testa properties is the main dormancy factor.
Therefore, as a step in the direction for agricultural use of this species it is reasonable to suggest the development of improved techniques for mechanical scarification as the dormancy must be overcome before the seeds of *A. cicer* can be used in lay farming systems or rangeland.

Autohinhibition is a type of intraspecific allelopathy, that a plant species inhibits the growth of its own kind through the release of some chemicals into the environment. The indication for this property has also been found for *A. cicer*. Inhibitors of germination are found in many higher plants. They can occur in vegetative organs but also in fruits and seeds (SAVELKOUL et al., 1992). Substances liberated from seeds during germination can influence other seeds. *A. cicer* causes an early browning of medium two hours after onset of incubation of the scarified seeds. The compounds obviously were not derived only from the testa, but resulted from the contact of the germination medium with the opened seed. Seedling root growth was delayed by this germination fluid and root surface browning became visible. These results refer to an autoinhibition of *A. cicer* during germination and early vegetative growth. This seed character has been documented for many species (SAVELKOUL et al., 1992; VAN STADEN and GROBBELAAR, 1995; KUSHIMA et al., 1998; SUMAN et al., 2002; QADERI et al., 2003). It should be tested if there is a threshold concentration, i.e. if a high number of seeds per given volume of liquid gives rise to a high inhibitory action.

PPO activity

Occurrence of PPO in dormant seeds and changes of the enzyme activity in the course of germination and seedling development has been intensively studied in wheat (DEMEKE et al., 2001; CHANG et al., 2006) and in tomato (MAKI and MOROHASHI, 2006).

DEMEKE et al., (2001) measured an increase in PPO assay activity for wheat seeds that imbibed water for 8-16 hours and a general decline in PPO assay activity after 16 hours. They showed that PPO activity increases for slightly abraded seeds. KOCAÇALISKAN et al., (1995) reported that during germination PPO activities of six plant species seeds including wheat did not present similar activity changes, but the activities in embryos were higher than in storage tissues. Excised coleoptiles and roots of wheat have been shown to own high PPO activity (TANEJA and SACHAR, 1974). The existence of PPO in

testas, its induction in endosperm and the rising activity in growing seedlings seem to show a general feature in the germination process. In any case, the occurrence of a preformed PPO in the testa and an induced form in the germinating seed point out, that there are different processes connected with the PPO activity. In general, the testa obviously fulfils an active part during early phase of germination or the final phase of seed development. This at least may be deduced from the occurrence of active enzymes in this plant structures (ANISZEWSKI et al., 2006).

CONSTABEL et al., (2000) found that PPO activity increased in wounded and unwounded leaves on wounded hybrid poplar plants over one to two days, respectively, and he considered the induction as a general plant response to wounding.

The manually operated scarification of *A. cicer* seed testas facilitated water uptake and consequently caused a rapid initiation of germination. Water uptake into the cells of dry seeds during germination results in temporary structural perturbations, particularly to membranes, furthermore, preformed mRNAs are available in dry embryos, new mRNAs are transcribed as regulatory processes during germination (BEWLEY, 1997). In many studies it has been reported that expression of PPO (e.g. in tomato CONSTABEL et al., 1995; potato THIPYAPONG et al., 1995; hybrid poplar CONSTABEL et al., 2000; pineapple STEWART et al., 2001) is arranged at the level of transcription activity of mRNA.

It is to consider that the existence of PPO in the testa layer is due to an early gen expression and enzyme synthesis which took place during the seed formation phase, whereas its high level in embryos and the increasing activity in developing seedlings will be under regulation of the seedling development programs, which are realized by germination related regulatory processes. It cannot be excluded, that also injury dependent reactions superimpose the occurrence of PPO in these tissues.

Anyhow, is known that plants need to contain PPO for protection against pathogens and herbivores (BASHAN et al., 1985; THIPYAPONG et al., 1995; GOODING et al., 2001; CONSTABEL et al., 2000; MAYER, 2006; MAKI and MOROHASHI, 2006). This may be the reason why young plants accumulate much PPO in roots to use it as a defense component against soil borne pathogens. It is to consider that the changes in PPO activity during germination are part of a developmental strategy of plants for their survival, but instead, it is also possible that it is a response to injury caused by scarification.

The activity of PPO in light cultured embryos and seedlings was higher than that of dark cultured embryos and seedlings. This may be seen in the context that PPO often is synthesized in early organ growth phases and is stored in a latent state in thylakoid membranes of the chloroplasts, from which it is liberated spontaneously and in an active status upon tissue injury (LIEBEREI and BIEHL, 1976; LAX and VAUGHN, 1991).

Our study has shown that the mechanic scarification of *A. cicer* seeds provides a suitable method to break seed dormancy. The findings also indicate that the PPO activity is an important factor during germination as well as during early plant growth. Activity of this enzyme in the testa, cotyledons, hypocotyl, roots and seedlings suggests its vital protective (protectional) role in establishing a new generation of plants. It will be necessary to analyse the system in much more detail, because a high PPO activity during germination and early growth may provide good protection, on the other hand a high PPO activity in vegetative tissue used for fodder may cause losses in fodder quality due to high chinone tanning and precipitation of the fodder protein.

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Address of the authors:

R. Lieberei, R. Sahbaz, University of Hamburg, Ecology and Biology of Useful Plants, Biocenter Klein Flottbek, Ohnhorststr. 18, D-22609 Hamburg, Germany

T. Aniszewski, University of Joensuu, Research and Teaching Laboratory of Applied Botany, Faculty of Biosciences, PO Box 111, 80101 Joensuu, Finland