Studies on *xHaynaldoticum sardoum* Meletti et Onnis metabolism during seed life-span: α-amylase and glutamate decarboxylase activity[1]

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Summary. Embryo GAD activity and α-amylase in the endosperm of 2 different physiological lines (CP; CV) of *xHaynaldoticum sardoum* Meletti et Onnis were evaluated and different stages of seed ripening and progressively older seeds were examined. Results concerning GAD activity during ripening show differences between CP and CV seeds, the former being more active. In the ageing seeds, the GAD remains constant (CP is twice as much as CV) up to 4th year and greatly decreases at the 5th. The α-amylase activity is fairly constant during ripening in CV endosperm and increases in CP: at the fully-ripe stage, both show similar values. During seed ageing, the activity decreases progressively in CV endosperm while, in CP, values are greater but fairly constant. The results are discussed in connection with dormancy and the different physiological ageing of seeds.

xHaynaldoticum sardoum Meletti et Onnis is a spontaneous hexaploid wheat to be found in the Mediterranean area and its female and male parents are *Triticum durum* and *Haynaldia villosa*, respectively[3]. Studies conducted by Meletti[4,5] have isolated 2 natural populations characterized by a solid (CP) or a hollow stem (CV) (CP and CV, in accord to Onnis[4]). Besides morphological and structural differences[6,7], caryopses from CP and CV populations also have the following physiological differences: CP is a winter-like wheat, with deep dormancy and a long dry after-ripening; CV is a spring-like wheat, with light dormancy and a short dry after-ripening.

Our previous experiments on *Triticum durum* led us to study certain of the metabolic characteristics of CV and CP populations of *xHaynaldoticum sardoum* with the aim of correlating their different physiological behaviour with the activity of 2 enzymes, glutamic acid decarboxylase (GAD; E C 4.1.1.15) and α-amylase (E C 3.2.1.1.), involved in the energetic and degradative metabolism of germinating seed[8–10]. In this way the pattern and the activity of the GAD and α-amylase during seed ripening and in the period after ripening up to the loss of germination capacity, were studied in the present paper.

Materials and methods. Caryopses (seeds) from CP and CV *xHaynaldoticum sardoum* Meletti et Onnis at different stages (milky, mealy, doughy, waxy, fully ripe[11]) were collected during the late spring and early summer of 1979. Calibrated seeds from crops from 1978, 1977, 1976, 1975, 1974 stored at laboratory conditions (18° ± 2 °C) in sealed glass containers, were also utilized. Germination assays of unripe, ripe and overly ripe seeds were carried out on water-moistened filter paper in Petri dishes, at 23 °C in darkness. The water content (relative humidity, %) of the seeds was evaluated by means of an Ultrax hygrometric balance. All the seeds were sterilized for 5 min with 1% NaClO solution and their embryos or endosperms were isolated by means of a sharp gauge and utilized as an enzymatic source.

The GAD was extracted by means of the method devised by Galleschi et al.[12], on batches of 350 isolated embryos. Pyridoxal-5-phosphate (PLP; 50 µl of 4 mM solution) was added to the standard reaction mixture as coenzyme.

The α-amylase activity was evaluated on isolated endosperms (20 for each of 5 replicates) homogenized with cold water (10 ml) and centrifuged at 6500 × g for 10 min at 4 °C. The supernatant (600 µl plus 0.2% $CaCl_2$) precipitated at 65 °C for 1 min was used as an enzymatic source. The α-amylase activity was evaluated by means of the method devised by Bernfeld[13] and modified by Dure[14] and expressed as mg maltose/min/endosperm. The maltose released was measured at 575 nm by a spectrophotometer.

The respiratory activity of the seeds and seedlings was measured at 30 °C by manometrically determining the oxygen uptake[15]. The materials were placed in the main body of the flask which contained 3 ml phosphate buffer (0.05 M, 7.2 pH) together with the central cell containing filter paper dipped in 0.4 ml of 10% KOH for CO_2 absorption.

The proteins, extracted by means of pH 4.0 sodium-acetate buffer and dialyzed overnight, were evaluated using the method devised by Lowry et al.[16].

Results and discussion. Gemination of unripe CP and CV seeds, evaluated after 10 days, shows very low germination energy (table): they show no germination until the doughy ripe stage and very low germination when at the waxy stage. At the fully-ripe stage, germination greatly increases

Relative humidity, respiratory activity and germination capacity of CP and CV ripening seeds. Seedling growth, at the 10th day, of fully ripe seeds

Ripening stages of seeds	Relative humidity (%)		Respiratory activity (µlO_2/30 min seed)		Germination % (at 10 days)	
	CP	CV	CP	CV	CP	CV
Milky-ripe	67	73	1.8	3.5	0	0
Mealy-ripe	56	63	2.1	3.8	0	0
Doughy-ripe	48	52	2.5	3.7	0	0
Waxy-ripe	41	42	2.2	3.2	4	5
Fully-ripe	12	12	0.9	0.9	70°	76°
Water imbibed ripe seeds	60	60	4.3	8.2	70°	76°

Seedling growth (cm) = CP, shoot 4.2; root – 6.4 CV; shoot 6.2; root 10.5.

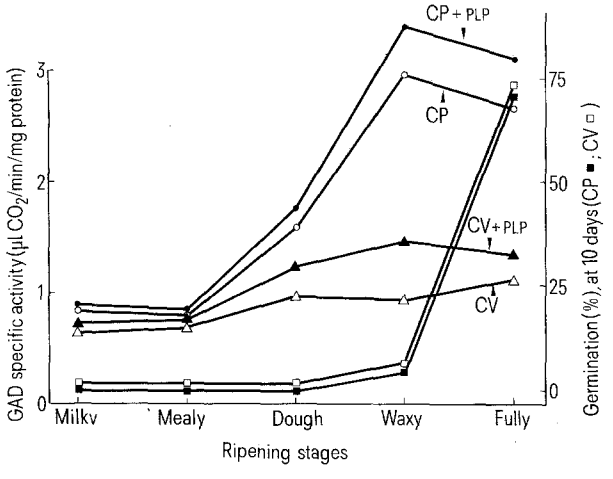

Figure 1. Embryo GAD specific activity and seed germination, after 10 days, during ripening.

and there are small differences between the CP and the CV seeds. During ripening the relative humidity of both seeds decreases progressively, the water content of the CV seeds being a little higher than that of the CP seeds: from the waxy stage on, the relative humidity of both kinds of seeds is similar. The respiratory activity of the CV seeds is higher than that of the CP seeds: it remains fairly constant up to the waxy-ripe stage and decreases at the fully-ripe stage. The respiratory activity of imbibing ripe seeds is greater and differences exist between CP and CV, the latter showing the higher value. The data are in accordance with the deeper relative dormancy of the CP seeds[7] in comparison with the CV seeds. This dormancy is also capable of depressing seedling growth (table).

During seed ripening the glutamic acid decarboxylase is very low at the milky and mealy stages and no differences exist between CV and CP. The enzymatic activity increases at the doughy ripe stage in both seeds: thereafter, it remains constant at low values in the CV embryos while greatly increasing in the CP embryos; PLP added to the reaction mixture does not increase the GAD activity at the 1st 2 stages, but is capable of improving it at the later stages without there being any differences between CV and CP embryos (fig. 1).

In the early dry storage period, the germination capacity of both CP and CV seeds is similar (about 100%; fig. 2); it remains constant during the 1st 4 years and then drops to 0% when the seeds are 5 years old.

The GAD activity is very different in CP and CV embryos: even though it remains constant during storage up to the 4th year and decreases to minimum values in the 5th year in both seeds, CP embryos show greater GAD activity (about twice as much as compared to that in CV embryos; fig. 2).

α-Amylase in the endosperms of ripening seeds was evaluated and experimental results show different patterns of enzymatic activity in the case of CP and CV seeds. At the milky stage, α-amylase activity is greater in CV than in CP: in the former, it increases slowly and maintains similar values in the subsequent phases of ripening; in the latter, the α-amylase activity increases greatly from the milky to the mealy stage, thereafter remaining fairly constant up to the waxy stage. In CP endosperms the activity decreases later when the seeds are fully ripened. At this stage of ripening, both CP and CV show similar values of α-amylase activity (fig. 3).

During seed storage, the α-amylase activity of CV endosperms decreases progressively and reaches the lowest values when the seeds loose their germination capacity. α-Amylase in CP decreases slightly in the 2nd year of storage and then remains fairly constant (the values are higher than in the case of CV) in the subsequent period up to the loss of germinability (fig. 4).

xHaynaldoticum sardoum Meletti et Onnis is a particular, spontaneous Mediterranean wheat whose caryopses, seedlings and plants differ morphologically, structurally and physiologically[3,6]. The present data also show there exist functional and enzymatic differences both between the 2 CP and CV 'morphological' lines and between *xHaynaldoticum sardoum* and its female parent *Triticum durum.*

The GAD activity, which was low in the early ripening stages of both seeds, later differentiates the 2 lines, the CP seeds showing greater (about twice as much) activity than in the case of the CV, from the mean ripe stage up to fully ripening. This pattern of GAD activity in the CP embryos appears to be similar to that to be found in *Triticum durum*[17], even though the values of specific activity are lower.

This activity does not appear to be correlated to the germination capacity since the percentage of germination is similar in both CP and CV. The improvement of the GAD by adding exogenous PLP, seems to be dependent on the ripening stage: in the early stages it appears to be insensi-

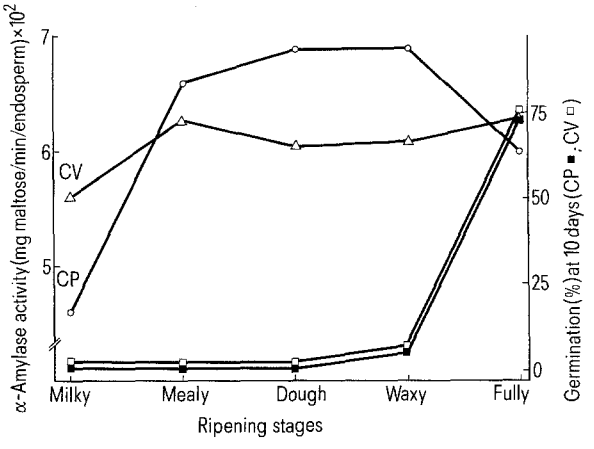

Figure 3. Endosperm α-amylase activity and seed germination, after 10 days, during ripening.

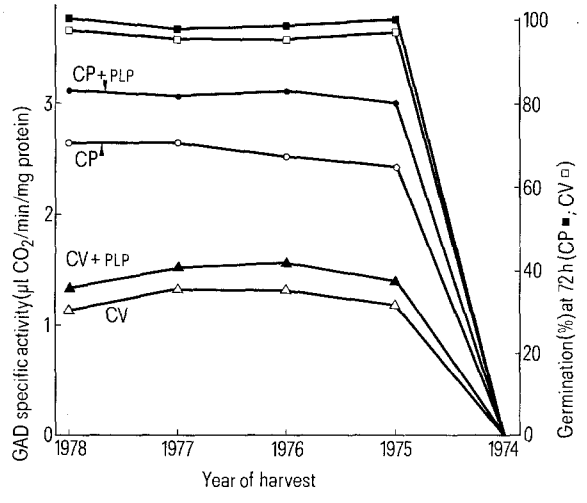

Figure 2. Embryo GAD specific activity and germination, during a 3-day period, in ageing seeds.

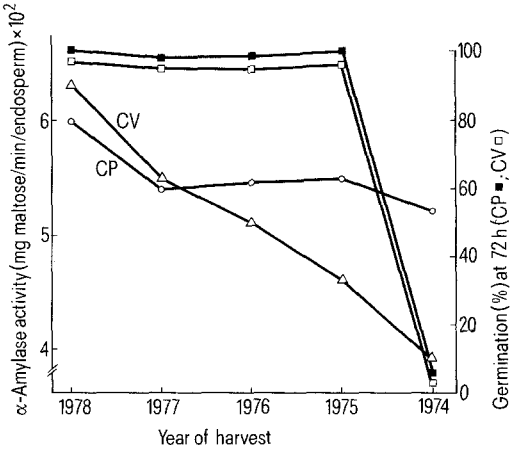

Figure 4. Endosperm α-amylase activity and germination, during a 3-day period, in ageing seeds.

tive to treatment compared to what happens in the subsequent stages. This hypothesis might be explained by possible structural variations in the enzymatic proteins or by the relative PLP concentration in the crude extract because of the difference in the hydratation of unripe and ripe materials.

During storage, CP and CV germination is similar and remains fairly constant for the 1st 4 years: on the other hand, the differences in the GAD activity are very pronounced, the level of activity being higher in CP embryos than in CV embryos. When both seeds are unable to germinate, this enzymatic activity also ceases: therefore this activity appears to be directly correlated to the germination capacity of seeds and their functional activity. This pattern also tallies with that of *T. durum* during seed ageing[10], even if the lifespan of *durum* wheat is possibly longer.

The addition of PLP improves the GAD activity of CP embryos more than that of CV embryos, thus pointing to a possible difference in the reactivation of the GAD of CP and CV embryos during storage. PLP treatment, however, is incapable of modifying the harmful effects of ageing also to be seen in the loss of germination capacity when seeds are older.

The pattern of the α-amylase activity both during seed ripening and during ageing makes it possible to differentiate between CP and CV seeds. Moreover, the differences to be seen in this enzymatic activity do not appear to have any effect as to germination during ripening and seed ageing. The increase in the amylolytic activity which occurs in CP endosperm is not accompanied, during ripening, by an improvement in germination energy. This confirms the finding that germination energy in ripening seeds is dependent upon growth regulators[18] more than upon the fact that storage products are made available to the embryo by the amylolytic activity. Similar conclusions may be drawn from the values of α-amylase in the endosperm during ageing. CP seeds, which loose their germinability in the 5th year of storage, show the same α-amylase activity when in dry conditions as well-germinating younger seeds do, and the α-amylase activity decreases in CV independently of the vigour of the seeds involved.

1 This work was supported by a grant from the 'Consiglio Nazionale delle Ricerche' (Rome, Italy).
2 Acknowledgments. The authors wish to thank Mr F. Saviozzi, Mr V. Sbrana and Mr R. Bertini for their expert technical assistance.
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Methods of metal incorporation into intracellular granules[1]

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Summary. The hepatopancreas of the garden snail *(Helix aspersa)* contains basophil cells which produce intracellular granules of $CaMgP_2O_7$. A variety of metals are incorporated into these granules either by direct substitution or by the synthesis of new pyrophosphate material.

Recent studies have shown that in virtually every group of invertebrate animals there are cells capable of producing intracellular inorganic granules[2-4]. These granules frequently contain a wide variety of environmentally available metals and therefore have often been interpreted as part of a cellular detoxification process[5].

In the common garden snail *(Helix aspersa)* pollutant metals become concentrated in the hepatopancreas tissue[6,7] and subsequent studies have identified the basophil cell as the site of this activity[8]. A variety of metals are taken up by these cells[9] and incorporated into granules which have been shown to be largely $CaMgP_2O_7$[10]. Pyrophosphates are in many ways ideally suited for such a cellular detoxification system for they are produced in large quantities by most anabolic processes and form extremely insoluble salts with a wide variety of metal ions[10]. The mechanisms whereby metals are deposited in these granules are not known but virtually all known examples of these concretions have been shown to be amorphous to X-rays. It is known that Mn^{2+} is incorporated into these granules extremely rapidly[9] and it was therefore decided to study the mechanism of metal incorporation by using this ion as a probe.

The snail *H. aspersa* was collected locally and used throughout this work. The animals were maintained in the laboratory on a diet of carrots, lettuce, cabbage and $CaCO_3$ powder for at least 2 weeks before use. Starved animals were left without food for 2-3 days. Three intravascular injections of 200 µl of 100 mM $MnCl_2$ in snail saline were given over a period of 30 h via a cannulated optic tentacle[9]. Granules were extracted from the hepatopancreas of the snail 24 h after injection by homogenizing the tissue and centrifuging the material in distilled water until a clean white deposit was obtained. The granules were spread on a grid and examined in a JEOL 100S electron microscope with ASID 5 using SEM. X-ray microprobe studies were performed on the same instrument using a Kevex detector and a Link analyser.

Starved snails will clear their blood of an injection of ^{54}Mn within 6-12 h. Most of this material can be recovered from the hepatopancreas of these animals and X-ray microprobe