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Short Communication

Amino Acids Content in Germinating Seeds and Seedlings from *Castanea sativa* L.

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ABSTRACT

During germination the chestnut (*Castanea sativa* L.) var ecotype 33 accumulates a large amount of asparagine in the cotyledons. This compound also accumulates in the growing axis:shoots and roots. In the cotyledons, γ-aminobutyrate (GABA) represents a major amino compound during germination and early seedling growth. In young seedlings, 35 days old, arginine predominates over the other soluble amino acids, particularly in roots. Five enzymic activities involved in arginine and GABA have been measured in the storage organ of the seed: arginase and ornithine carbamyltransferase decrease during germination indicating the slowing down of the urea cycle. In contrast, ornithine aminotransferase increases. Glutamate decarboxylase is particularly active about 21 days after imbibition and GABA aminotransferase activity decreases during germination. These two activities are in good agreement with the likely transport of GABA from cotyledons to growing axis. Asparagine, arginine, and GABA are the three amino compounds obviously involved in the mobilization of nitrogen reserves in the germinating chestnut seeds *Castanea sativa*.

The significance of a high level of GABA[1] in chestnut seeds (*Castanea sativa* L.) (10) is not yet understood. Several reports of its accumulation, under stress, in higher plants have been made (5, 24, 26). It was proposed as a temporary storage product (5, 21).

Much work has been published on transport and metabolism of nitrogen compounds in germinating seeds (15–18). The cotyledon can be regarded as a reserve organ which is utilized during germination to support the growth of the axis tissue. Seed germination is accompanied by marked changes in the reserve constituents: the free amino acids and those released by the breakdown of reserve proteins may be used as an energy source or are transported to the growing axis where they are metabolized or accumulate (16).

The present paper reports on changes in free amino acids pool in the cotyledons and the growing axis of germinating chestnut at three stages of development. Enzymic activities implied in the metabolism of arginine, ornithine, and GABA are studied as a start toward the elucidation of their role in the regulation of nitrogen nutrition.

[1] Abbreviations: GABA: γ-aminobutyric acid; OAT: ornithine amino transferase; α keto glu: α-ketoglutaric acid; OCT: ornithine carbamyl transferase; Cit: citrulline.

MATERIALS AND METHODS

Plant Materials. Chestnut seeds (*Castanea sativa* ecotype 33) were collected in October 1983 and preserved at +4°C (Station de Recherche d'arboriculture fruitière 'La Grande Ferrade' 33140 Pont de la Maye. Institut National de la Recherche Agronomique). They were sown in moist perlite at +20°C. Variability in seed germination and seedling growth was large, therefore the age of seedling was defined in terms of apparent developmental stages of growth corresponding approximately to age after imbibition: stage 1 (10 chestnuts): 7 d after imbibition, embryo (root) out of the seed 1 cm; stage 2 (10 chestnuts): about 21 d after imbibition, seedling 10 to 15 cm, shoots and roots well spread: shoot with 2 leaflets; stage 3 (10 chestnuts): about 35 d after imbibition, seedlings 20 to 22 cm, shoot with 2 leaves spread and 4 leaflets.

Seed coats were discarded, plant parts divided as cotyledons, roots, shoots, and then preserved by freezing (−20°C) before use.

Free Amino Acids, Amides (Asn, Gln) Extraction and Determination. Extraction was performed by crushing plant material twice in 10 mM HCl in the ratio 20% w/v. Amino acids determination was performed as described previously (9). Total amino acids content was expressed as μmol/g fresh tissue. Data are the results of duplicate analysis. Amino acids composition was expressed as molar percentage of total. Because of its interference in the ninhydrin reaction, ammonia was determined as NH_4Cl but exact determination of ammonia was not estimated since it may originate from hydrolysis of glutamine or asparagine which occurs during the analytical procedure.

Enzyme Assays. All enzymes activities were assayed at 30°C. The unit of enzymic activity is the nanokatal: degradation of 1 nanomol of substrate/s·g of plant tissue under the stated assay conditions. Each enzymic activity was measured by change of amino acids concentration after 1 h activity: for each measure 2 mixtures were prepared: one inactivated by TCA before addition of plant extract, the other after 1 h reaction with enzyme (plant extract).

Plant extraction was performed by crushing an aliquot of plant tissue from 10 chestnut cotyledons (5–10 g) in 100 mM Hepes buffer (25% w/v) adjusted to optimal pH of each enzyme activity.

Substrates were solubilized in 100 mM Hepes buffer; pH values were adjusted according to optimal pH of each enzymic reaction with NaOH or HCl. For 1 ml substrate, concentrations were as follows:

Arginase: (EC 3.5.3.1.) pH = 9.4; Arg: 20 μmol, $MnCl_2$ 1 μmol.

OAT: (EC 2.6.1.13) pH = 8; Orn 20 μmol, α-ketoglutarate: 10 μmol, pyridoxal-P: 0.1 μmol.

OCT: (EC 2.1.3.3.) pH = 7.14; Orn: 20 μmol, carbamyl-P: 10

Table I. *Free Amino Acids*

Total amino acid determination was performed by ninhydrin colored reaction on duplicate analysis.

Stage	Free Amino Acids Expressed as Norleucine		
	Cotyledons	Embryo	
		μmol/g of plant	
1	56.9	26.6	
		Shoot	Root
2	50	36.8	26
3	36.2	53.8	56.1

μmol.

Glutamate decarboxylase: (EC 4.1.1.15) pH = 5; Glu: 20 μmol, pyridoxal-P: 0.1 μmol.

GABA aminotransferase: (EC 2.6.1.19) pH = 8.2; GABA: 20 μmol, keto glutarate: 10 μmol, pyridoxal-P: 0.1 μmol.

Final reaction mixtures were: substrate: 1 ml; plant extract: 1 ml; TCA (20% w/v): 1 ml (added after 1 h reaction).

Amino acids determination was performed with an animo acid analyzer (Technicon NC 2P).

Arginase activity was calculated from the quantity of formed ornithine. OCT activity was measured by the quantity of formed citrulline. Glutamate decarboxylase activity was measured by the increase in GABA. To avoid possible interference with other transaminases, OAT was measured by decrease in ornithine concentration and GABA aminotransferase by decrease in GABA concentration.

RESULTS AND DISCUSSION

Free Amino Nitrogen. In germinating chestnut cotyledons (Table I) the pool of free amino nitrogen decreases from stage one to stage three. In the growing axis it varies in the reverse order: in roots and shoots it is highest in the third stage. Since these chestnuts were germinated without an exogenous source of nitrogen, the seed reserve proteins and free amino acids were the only source of nitrogen for the growing axis: the changes in the free nitrogen distribution show the gain by the axis reflecting the loss by the cotyledons.

Asparagine, Arginine, and GABA. Table II shows the high accumulation of asparagine from stage one to stage three in the cotyledons: 4, 24, then 30% of the total. It has been suggested (4) that amino acids in early stage of germination rapidly provide 2-keto acids with a release of ammonia accompanied by a biosynthesis of high nitrogen compounds particularly amides, stored and transported to growing axis. In the young chestnut seedling a preponderant part is also reserved to asparagine: 30, 32, 30% in roots and a higher level in shoots 46% in stage two, 44% in stage three. The data on asparagine imply a major role as storage and transport compound in chestnut as in pea plant (12) and soybean seed (19).

These results show that asparagine is a key element in the chestnut seed metabolism both during germination and maturation (9) as reported for lupin (1) and for cotton seed (11).

An important role of arginine in the metabolism of the seedling has been claimed (2). The level of arginine is high in chestnut cotyledons in stage one: 10%. It decreases gradually to stage three in the cotyledon whereas it increases in the growing tissues to reach 24% in shoots and 35% in roots in stage three. This compound may be metabolized with release of ammonia in early stage of germination by arginase (25) and urease (unpublished results) present in chestnuts cotyledons. During seed germination

Table II. *Stages of Germination Are Defined in Terms of Apparent Development of Growth Corresponding Approximately to Age after Imbibition 7, 21, 35 Days after Imbibition*

Amino acids are determined with Technicon NC 2P as described previously (9). Ammonia includes that from hydrolysis of amides during the analytical procedure. It is expressed as NH_4Cl because of its interference in the ninhydrin colored reaction which is used to determine total amino acids.

	Stage 1		Stage 2			Stage 3		
	Cotyledons	Embryo	Cotyledons	Shoot	Root	Cotyledons	Shoot	Root
Cysteic acid	0.6	0.25	0.08	0.36	0.44	0.13	0.38	0.3
ASP	3.38	4.33	3.27	2.84	3.26	3.25	4.58	2.9
THR	0.76	0.6	2.3	0.72	0.79	2.43	1.7	0.84
SER	2.33	1.76	4.83	2.59	1.32	6.25	1.85	0.88
ASN	4.17	30.4	24.1	46.5	32	30.1	44.4	30
GLU	11.6	3.8	0.91	3.2	2	2.84	3	2.39
GLN	2.95	1.14	1.21	1.21	1.55	0.49	0.39	0.74
GLY	1.9	0.43	1.79	0.48	0.32	1.81	0.28	0.44
ALA	8.4	1.6	6.37	1.98	1.35	6.18	1.15	0.91
VAL	4.29	0.54	3.04	0.47	0.72	2.26	0.37	0.47
Unknown expressed as norleu	0.14	0.27	0.13	0.05	0.07	0.10	0.20	0.30
MET	0.15	0.03	0.35	0.37	0.3	0.3	0.29	0.12
ILE	2	0.21	1.21	0.21	0.48	0.61	0.18	0.12
LEU	1.59	0.23	0.93	0.14	0.36	0.3	0.15	0.06
TYR	0.68	0.07	0.62	0.139	0.23	0.45	0.52	0.13
PHE	1.19	0.21	0.84	0.18	0.55	1.55	0.24	0.18
GABA	25.25	2.28	22.1	2.23	4.46	20.8	0.91	1.27
TRP	0.48	0.47	1.5	0.27	0.01	2.30	0.56	2.31
NH_4Cl	16.2	43	12.8	29.7	25.6	12.5	13.4	16.5
ORN	0.14	0.47	0.15	0.31	0.32	0.17	0.05	0.17
LYS	0.25	0.24	0.4	0.23	0.58	0.19	0.19	0.35
HIS	0.91	0.41	1.75	0.48	0.74	1.21	1.42	2.8
ARG	10.5	7.26	9.1	5.3	22.6	3.67	23.8	35.6

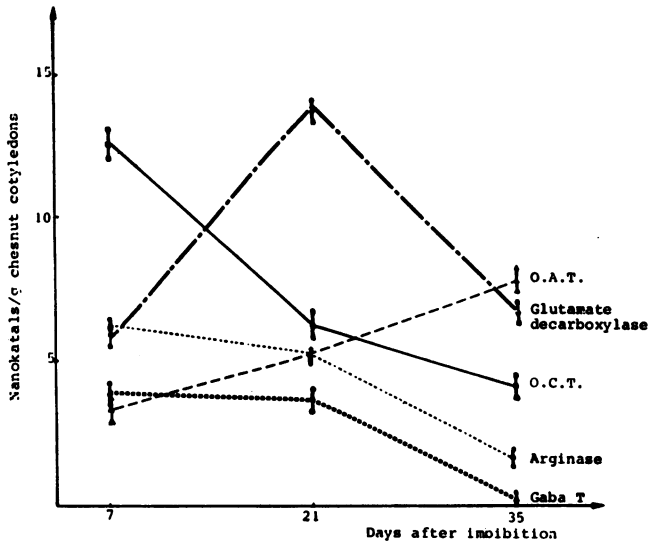
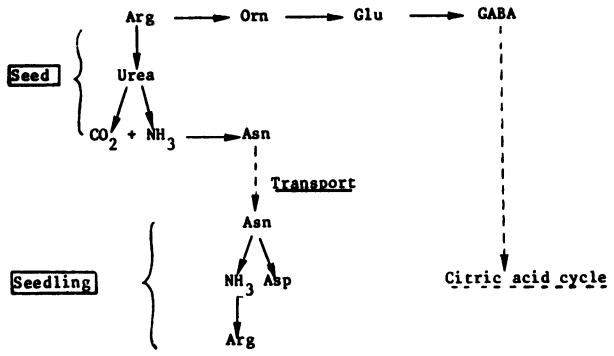


FIG. 1. Enzymic activities at three stages of germination in chestnut cotyledons: 7, 21, and 35 d after imbibition. Standard reaction mixtures were buffered according to the enzymic activities measured: arginase: pH = 9.4; OAT: pH = 8; OCT: pH = 7.14; glutamate decarboxylase: pH = 5; Gaba T (aminotransferase): pH = 8.2. Specific activities are expressed as nanomol of amino acid metabolized during 1 s by 1 g of plant tissue.



SCHEME 1

it may be either (16) directly translocated to the growing axis or rather degraded *in situ*. The *in situ* degradation by arginase has been shown in broad bean (14), in pumpkins (22), in peas (6, 7) and in grapevine (20): arginase increases rapidly in the first d of germination, decreasing thereafter. In germinating chestnut cotyledons arginine, arginase and OCT decrease in the three stages but it must be pointed out that in our experiment the first stage is the 7th d after imbibition. It has been shown that arginine was metabolized to ornithine, glutamate, and GABA (3) which was then translocated to the axis were it was metabolized to citric acid cycle acids, in germinating pumpkin cotyledons. Recently it has been shown in pea seeds (6, 7) that arginine synthetizing enzymes activity is low during the germination process compared with the activity in the developing seed: the presence of these enzymes in germinating seeds might be a "remnant of an enzymic machinery used in the developing seed for the synthesis of arginine (6)." However, arginase and OAT (7) rapidly increased during the first d of germination, then decreased; urease increased later and for a longer time. OCT might have in pea seedling (8) an anabolic function in the synthesis of citrulline.

The simultaneous presence of arginase and OCT in germinating chestnut cotyledons might be considered as a regulation process to assure availability of arginine. This compound would be a temporary nitrogen storage compound before its degradation via the arginase, urease pathway. The data on OAT in germinating chestnuts implies its utilization by the metabolic route previously described in germinating pumpkin seeds (3). An increase of activity of OAT in early stage of germination has been demonstrated in pea seeds (7) and also observed in cotyledons of pumpkins seedling (23).

In chestnut seeds, GABA has been shown to represent an important part of the free amino nitrogen, particularly in *Castanea sativa* (10). During the germination it remains at a high level.

There are several reports of an increase of this compound in plants under some stress such as anoxia (24), abrupt transfer to lower temperature or mechanical manipulation (26), in response to viral attack (5) and to light change (21). In the latter two observations GABA has been proposed as a temporary storage product. In cultured rice cells it increases in response to ammonium and glutamine nutrition (13). In mature chestnut seeds, GABA accumulation varies according to the species (10). It is likely a temporary storage product and perhaps an energy transport form, since asparagine is the nitrogen transport form, from cotyledons to growing axis. Scheme 1 shows the metabolic route that is proposed in germinating chestnut seed of *C. sativa*.

This metabolic pathway may be of general occurrence in germinating seeds. It will be of particular interest to confirm this metabolic route in various seeds by use of radioactive molecules *in vivo*.

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