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TRIGONELLINE, NICOTINIC ACID AND NICOTINAMIDE IN SEEDLINGS OF *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; trigonelline; nicotinic acid; nicotinamide; pyridine nucleotide metabolic pathway; cell arrest in G2.

Abstract—Levels of trigonelline, nicotinic acid and nicotinamide have been examined in pea plants from the ungerminated seed to 10 days of age. The total quantity of trigonelline ranged between 70 and 81 μg in seeds to 7-day-old plants. At 10 days, the quantity of trigonelline increased to 101 μg. During the first 10 days, the concentration of trigonelline decreased in all regions of the seedling. Quantities of nicotinic acid within the seedling fell immediately after germination from 11.4 to 4.4 μg at 3 days but increased to 18.3 μg at 10 days. Unlike the decreasing trigonelline concentrations, nicotinic acid concentration throughout the seedling appeared relatively stable. Quantities of nicotinamide were less than 1% that of trigonelline, and never reached 1 μg total. The only noticeable increase in the concentration of nicotinamide occurred in leaves of 10-day-old plants. When [^{14}C]trigonelline was injected at the cotyledonary node, it was transported to all regions of the plant and the majority (65%) went to epicotyl tissues.

INTRODUCTION

Trigonelline functions as a plant hormone by promoting cell arrest in G2 in roots and shoots of *Pisum sativum* L. [1–3]. Trigonelline, nicotinic acid an essential vitamin, and nicotinamide function within the pyridine nucleotide pathway. Trigonelline was thought to be a storage form of nicotinic acid, because excessive amounts of nicotinic acid were considered to be toxic to plants. Even so, nicotinic acid is found in high levels in cultured roots [4]. The present experiments were undertaken to monitor levels of trigonelline, nicotinic acid and nicotinamide in seedlings, and to relate these levels to cell arrest in G2 and to determine the transport of trigonelline in young pea seedlings.

RESULTS

Quantities of trigonelline, nicotinic acid and nicotinamide in P. sativum

Concentrations and quantities of trigonelline, nicotinic acid and nicotinamide were determined in tissues from non-germinated seeds through to 10-day-old seedlings. These were related to promotion of cell arrest in G2 in root meristems. Proportions of cells arrested in G2 in the root meristem decreased with an increase in seedling age (Table 1). This decline in cell arrest in G2 was correlated with a decrease in concentration and quantity of trigonelline in roots (Fig. 1). Cotyledons are storage organs for trigonelline during early seedling growth (Fig. 1). Within the first 10 days of germination, *ca* 70% of the trigonelline present in dry seeds was transported to other plant tissues from 3 to 10 days. By day 7, the first internode contains *ca* 50% of the total trigonelline. The quantity of trigonelline in root meristems (0–10 mm) decreased during the first 10 days of growth, although the total amount of trigonelline in each meristem was less than 1 μg.

The total quantity of trigonelline from non-germinated seeds to 7-day-old seedlings ranged from 70 to 81 μg (Fig. 1), with a significant increase in trigonelline content observed in 10-day-old seedlings.

Nicotinic acid concentrations remained constant during early seedling growth (Fig. 2). There was a greater variation in concentrations of nicotinic acid than of

Table 1. Concentrations of trigonelline and proportions of cells arrested in G2 in stationary phase meristems of excised roots of *P. sativum* as a function of seedling age

Seedling age (days)	Trigonelline concentration (μg/g tissue)	Proportion of cells in G2
3	91	0.59
5	79	—
7	45	0.40
10	29	0.19

By regression analyses the correlation coefficient of proportion of cells arrested in G2 with trigonelline concentration was 0.95.

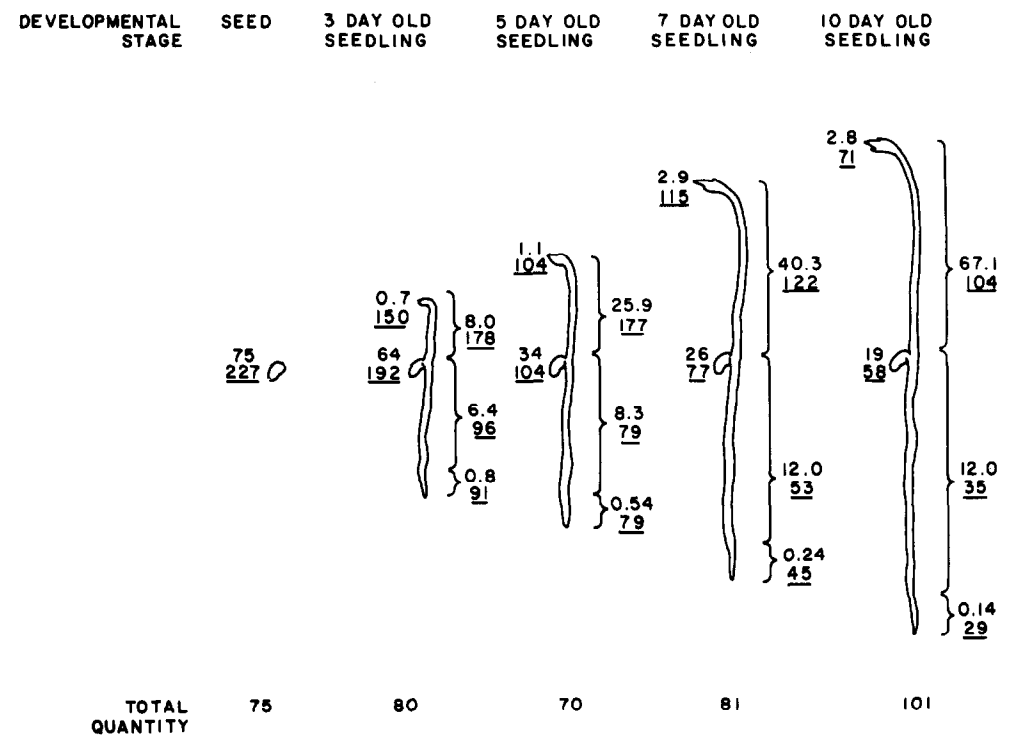

Fig. 1. Quantities (μg/portion) and concentrations (μg/g tissue) of trigonelline in seeds and seedlings of *Pisum sativum*. Quantities and concentrations are given by upper and lower values, respectively.

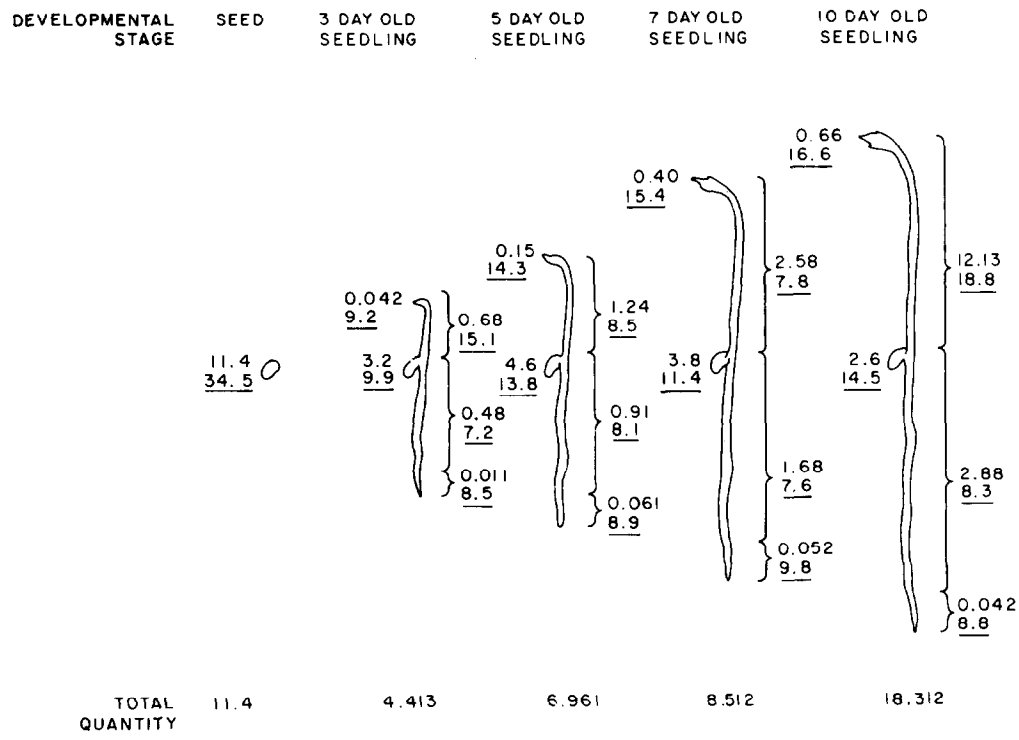

Fig. 2. Quantities (μg/portion) and concentrations (μg/g tissue) of nicotinic acid in seeds and seedlings of *Pisum sativum*. Quantities and concentrations are given by upper and lower values, respectively.

trigonelline in first internodes and primary leaves. Nicotinic acid concentrations were *ca* 10% of the trigonelline concentrations. No consistent decrease in nicotinic acid concentration occurred in root meristems as seedlings aged, suggesting there is no relationship between concentrations of nicotinic acid and proportions of cells in G2. *Ca* 11.4 μg of nicotinic acid was present in ungerminated seeds. *Ca* 4.4 μg of nicotinic acid was present in cotyledons of 3-day-old seedlings but the amount increased to 19.6 μg in 10-day-old seedlings. As the quantity of trigonelline increased from 7 to 10 days, there was an increase in nicotinic acid, the immediate precursor of trigonelline. The first internode contained *ca* 45% of all the nicotinic acid in 7-day-old plants and increased to 61% in 10-day-old plants (Fig. 1).

Quantities of nicotinamide were less than 1% of those of trigonelline (Fig. 3). Concentrations of nicotinamide in ungerminated seedlings were 0.03 μg/g tissue and increased 13-fold to 0.40 μg/g tissue in cotyledons of 3-day-old seedlings. The increase in nicotinamide concentration was correlated with a decrease in nicotinic acid concentration during the first 3 days of seedling growth. The concentration of nicotinamide in cotyledons varied from 0.34 μg/g in 4-day-old seedlings, to 0.18 μg/g tissue in 7-day-old seedlings.

Concentrations of nicotinamide in root meristems varied with seedling age. No correlation between nicotinamide concentration and cell arrest in G2 was present. At 10 days of age, mature root tissues had higher nicotinamide concentrations than younger seedlings. Stationary phase meristems from 10-day-old seedlings had few cells arrested in G2 [1].

In first internodal tissues, concentrations of nicotinamide decreased from 0.42 μg/g tissue in 3-day-old seedlings to 0.11 μg/g in 10-day-old seedlings. The greatest increase in nicotinamide concentration during early seedling development occurred in primary leaves (4.6 μg/g tissue in 3-day-old seedlings). The concentration of nicotinamide in primary leaves decreased to 1.5 μg/g at 5 days but increased to 11 μg/g in 10-day-old seedlings. A continual increase in nicotinamide occurred from ungerminated seeds to 10-day-old seedlings. The greatest increase occurred between 7 and 10 days. However, quantities of nicotinamide never reached 1 μg per plant.

The proportion of cells arrested in G2 in seedling roots was not correlated with concentrations or quantities of either nicotinic acid or nicotinamide. Of the seedling ages examined, the decrease in the proportion of cells arrested in G2 was better correlated with a decrease in trigonelline concentration than in quantity of trigonelline per root (Table 1).

Transport of trigonelline

Experiments were performed to determine the rate of transport and degree of metabolism of trigonelline in decotyledonized seedlings. After 24 hr exposure to [carboxy-^{14}C]trigonelline, 65, 24, 10 and 1% of the total radioactivity was present in epicotyls, injection area, mature root tissues and root meristems, respectively (Table 2). Between 65 and 73, 0.38 and 2.9% of the total radioactivity in all samples was present as trigonelline, nicotinic acid and nicotinamide, respectively. Besides trigonelline, the largest percentage of radioactivity (10–15%) was observed as NAD and a mixture of NADP and NMN. Between 11 and 20% of the radioactivity could not be attributed to members of the pyridine nucleotide pathway.

Most radioactive trigonelline was present in epicotyls (65%), only 10% was in roots, with the remainder (25%) in the injection region. The distribution of radioactivity among compounds, both known and unknown, was identical in all tissues.

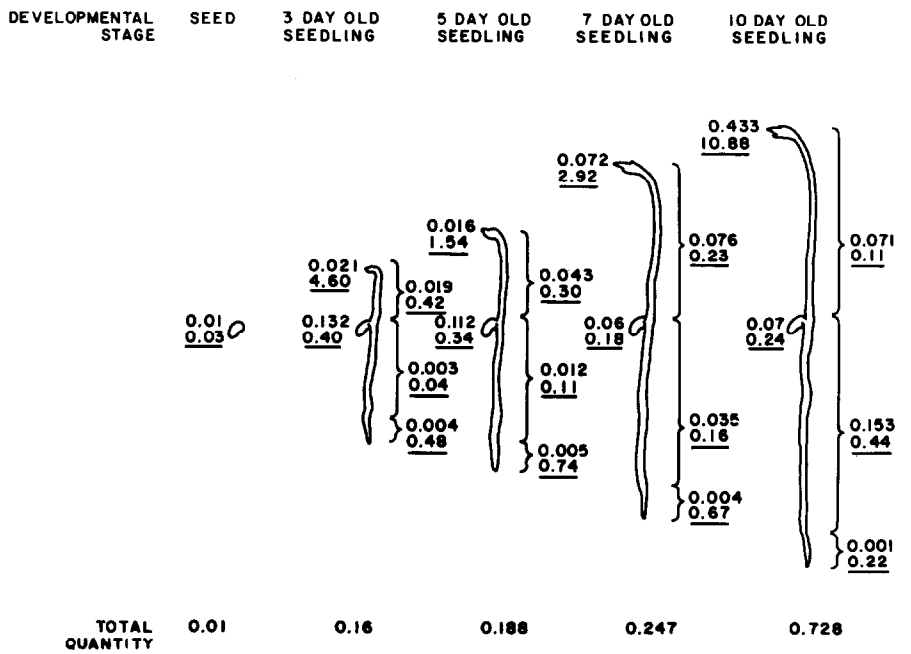

Fig. 3. Quantities (μg/portion) and concentrations (μg/g tissue) of nicotinamide in seeds and seedlings of *Pisum sativum*. Quantities and concentrations are given by upper and lower values, respectively.

Table 2. Percentage of radioactivity in various intermediates of the pyridine-nucleotide metabolic pathway in various plant portions when radioactive trigonelline was injected into the cotyledonary node of decotyledonized seedlings of *Pisum sativum*

Plant portion	Substance	Percentage of counts in portion	Percentage of counts in seedling
Epicotyl tissue	Trigonelline	70.40	45.96
	NAD	6.61	4.31
	NA	1.90	1.24
	NAM	0.38	0.25
	NADP/NMN	4.53	2.95
	Other§	16.18	10.54
	Total	100.00	65.25
Injection area*	Trigonelline	65.30	15.87
	NAD	7.10	1.73
	NA	2.93	0.71
	NAM	0.68	0.17
	NADP/NMN	3.53	0.86
	Other	20.46	4.97
	Total	100.00	24.31
Mature root†	Trigonelline	72.69	7.40
	NAD	8.26	0.84
	NA	0.52	0.05
	NAM	0.70	0.07
	NADP/NMN	6.12	0.62
	Other	11.71	1.19
	Total	100.00	10.17
Root meristem‡	Trigonelline	65.50	0.18
	NAD	5.50	0.01
	NA	0.27	0.00
	NAM	0.38	0.00
	NADP/NMN	11.60	0.03
	Other	16.75	0.05
	Total	100.00	0.27
Entire seedling	Trigonelline	—	69.41
	NAD	—	6.89
	NA	—	2.00
	NAM	—	0.49
	NADP/NMN	—	4.46
	Other	—	16.75
	Total	—	100.00

*Refers to the cotyledonary node region where the radioactive trigonelline was injected.

†Refers to root tissues between the cotyledonary node and the 0–10 mm root meristem.

‡Refers to 0–10 mm terminal root tissues.

§Refers to areas on TLC plates not specifically attributed to any of the six substances listed. Since each of the six substances listed did not migrate at the same relative mobility, most of the counts in the 'other' category were really found in the six substances listed. As a result the values for the six substances listed are conservative values.

DISCUSSION

Trigonelline quantities within seedlings of *P. sativum* appear relatively constant throughout the first 7 days of seedling growth. At 10 days the overall quantity of trigonelline increased. Cotyledons are the storage organs for trigonelline and these storage organs possess a finite amount. Trigonelline concentrations decrease throughout the plant during the first 10 days after germination, but the quantity only decreases in the meristematic root during this period, and as the quantity and concentration decrease so does the percentage of cells in G2. The majority of the trigonelline is transported to the epicotyl. Cell arrest in G2 also occurs in shoots [5].

Immediately after germination most nicotinic acid is converted to trigonelline. The concentration of nicotinic acid does not vary from 3- to 10-day-old seedlings suggesting that this essential vitamin was maintained at a constant level throughout the early development.

Nicotinamide was present at concentrations lower than nicotinic acid during early ontogeny. Concentrations of nicotinamide only increased in primary leaves during seedling development. A possible correlation could be attributed to high nicotinamide levels in photosynthetic organs. Godavari and Waygood [6] reported that when wheat leaves were fed either radioactive nicotinic acid or nicotinamide, *ca* 25% of the radiolabel existed as nicotinamide, suggesting that higher levels of nicotinamide rather than nicotinic acid were required by leaves.

When radioactive trigonelline is injected into 3-day-old seedlings NAD, NADP and NMN become labeled. These data show that trigonelline may be converted to other members of the pyridine nucleotide pathway. These results differ from results with cultured roots in which trigonelline is not converted to other members of the pathway [4]. The demand for NAD and NADP may be greater in intact seedlings than in isolated roots, and quantities of NAD and NADP would probably have been elevated if the seedlings had been exposed to light.

Different metabolic strategies seem to exist between excised roots and roots of intact seedlings. Excised roots retain most radioactivity as trigonelline whether in roots or media [4]. Intact seedlings demethylate trigonelline and convert it into nicotinic acid and other members of the pyridine nucleotide pathway, such as NAD and NADP.

Results show that trigonelline is transported from cotyledons to other portions during early seedling development. Trigonelline promotes cell arrest in G2 in root meristems during the first 10 days of growth but seems to be ineffective thereafter. This loss of effectiveness in intact seedlings is correlated with a decrease in the quantity of trigonelline in root meristems. The increase in trigonelline in 10-day-old seedlings is attributed to synthesis of trigonelline in epicotyls.

EXPERIMENTAL

General culture conditions. Seeds of *Pisum sativum* L. were surface sterilized with undiluted Clorox containing 5.25% NaOCl, stirred frequently for 10 min, washed with sterile H_2O and germinated in sterile vermiculite.

Bioassay for promotion of cell arrest in G2. General culture techniques to determine the ability of plant-derived extracts that promote cell arrest in G2 by a standardized bioassay have been described [5]. Under aseptic conditions excised root meristems were placed in culture medium with sucrose for 3 days before temporary carbohydrate deprivation (establishment of a stationary phase meristem). A stationary phase meristem may be defined as a meristem (0–2 mm portion) in which progression through the cell cycle has ceased temporarily [7]. In some expts an intermediate of the pyridine nucleotide metabolic pathway was added to 50 ml of sucrose medium which normally supports growth of 10 excised roots. With eventual establishment of a stationary phase by temporary carbohydrate deprivation, cells were arrested in G1 and in G2 (2C and 4C contents, respectively) within the terminal meristem. If roots from 3-day-old seedlings are placed in sucrose medium alone before establishment of stationary phase, only 0.20 cells arrest in G2. However, if a sufficient concn of trigonelline is present in medium with sucrose before establishment of stationary phase, then a larger proportion (*ca* 0.40–0.60) of cells arrest in G2 [5].

DNA measurements. Measurements of relative DNA per nucleus of Feulgen-stained nuclei were obtained by microfluorimetry. This method is modified from that of ref. [8] and is described elsewhere [9].

Nicotinic acid, nicotinamide and trigonelline concentrations. To determine the concns of nicotinic acid, nicotinamide and trigonelline in roots, 0–1 cm terminal root segments of 3-day-old roots were excised and extracted in an EtOH series [6]. Each extract was concd and spotted on Si gel TLC UV plates, 250 μm thickness (Analtech Corp. Newark, U.S.A.). For nicotinic acid and nicotinamide, extracts were developed in *iso*-PrOH–H_2O–HOAc (29:5:6). Plates were allowed to air dry and both nicotinic acid and nicotinamide were eluted with EtOH. This soln was evaporated to dryness and the residue resuspended in 0.5 ml H_2O and chromatographed (HOAc–Me_2CO–MeOH C_6H_6, 1:1:4:14). Nicotinamide and nicotinic acid were eluted individually and quantities of nicotinic acid and nicotinamide were determined by HPLC [10] using a Whatman C-18 reverse phase column with 1 mM KPi as solvent (pH 5.8), at a flow rate between 2 and 3 ml/min.

For trigonelline, extracts were developed in Me_2CO–H_2O (1:1). Plates were allowed to air dry and trigonelline was eluted. Quantities of trigonelline were determined by HPLC [10] using a Whatman Partisil-Sax 10 column using 7 mM KPi as solvent (pH 5.8) at a flow rate of 1 ml/min.

Transport of [carboxy-^{14}C]trigonelline. [Carboxy-^{14}C]trigonelline (4 μl) was injected into 3-day-old decotyledonized seedlings at the point of cotyledon excision. Seedlings were allowed to grow in 25% strength Hoagland soln [11] for 24 hr. Extracts were prepared of various tissues and extracts were chromatographed (*iso*BuOH–18 M NH_4OH–H_2O, 66:1:33). Nonradioactive standards were placed in extracts to identify bands. Bands were removed and counted by liquid scintillation.

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