# Trypsin Inhibitor Activity in Field Pea (*Pisum sativum* L.) and Grass Pea (*Lathyrus sativus* L.)

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Trypsin inhibitors in grain legumes decrease the digestibility of protein and cause pancreatic enlargement. Seed samples of 17 field pea cultivars grown at 5 locations and 9 grass pea lines grown at 2 locations in western Canada during 1993 and 1994 were analyzed for trypsin inhibitor activity (TIA). TIAs in field pea differed significantly among cultivars. Mean TIA in field pea ranged from 2.22 trypsin inhibitor unit (TIU) $mg^{-1}$ of dry matter (DM) for Danto to 7.66 TIU $mg^{-1}$ of DM for Baroness. Cultivar accounted for more of the total variability (55%) in field pea than environment (18%). TIAs in grass pea did not differ among cultivars or environments. The mean TIA was 27.51 TIU $mg^{-1}$ of DM for the grass pea lines tested. The correlation between the levels of TIA and seed yield was near zero in field pea and grass pea.

**Keywords:** *Field pea; grass pea; Lathyrus sativus L.; Pisum sativum L.; trypsin inhibitor activity*

## INTRODUCTION

Field pea (*Pisum sativum* L.) is an inexpensive source of protein and energy and consequently is an important part of human diets and animal feeds in many parts of the world. Field pea planting area in western Canada increased from 73 600 ha in 1985 to 840 000 ha in 1997. Field pea is now a major pulse crop in western Canada, which diversifies cropping options for cereal growers. Currently, approximately two-thirds of the field pea production in Canada is exported for animal feed; the largest buyer is the European compound feed industry. Efforts are underway in Canada to develop new export markets and to develop the domestic feed market.

Grass pea (*Lathyrus sativus* L.), chickling vetch, or khesari, a pulse crop with high yield potential and drought tolerance, is an essential food crop for animals and humans in some countries in western Asia and northern Africa (Spencer et al., 1986). It could become a useful rotation crop in the brown soil zone of the Canadian prairies, an area that lacks an adapted annual legume alternative. The first grass pea cultivar in Canada, X850002, was released recently by the Morden Research Centre.

Field pea and grass pea contain a number of naturally occurring compounds that interfere with nutrient availability and are thus designated antinutritional factors. These include tannins, alkaloids, saponins, glucosides, trypsin inhibitors, lipoxygenase, and antithyroid substances. The most important of these factors are protease inhibitors and tannins (Gatel and Grosjean, 1990). In addition, grass pea contains a neurotoxin, $\beta$-*N*-oxalyl-L-$\alpha$,$\beta$-diaminopropionic acid (ODAP), also known as $\beta$-*N*-oxalylamino-L-alanine (BOAA), which causes lathyrism in humans and animals (Chowdhury, 1988). The Morden Research Centre grass pea breeding program is producing only lines that are low in ODAP (<0.06%). High-ODAP lines grown in some Asian countries are considered dangerous for human consumption.

Trypsin is a proteolytic enzyme secreted by the pancreas. Trypsin inhibitors are present in many legumes in varying amounts. When trypsin is inhibited, proteins are not digested adequately and fewer amino acids are available for growth. It is known that many plant protease inhibitors can inactivate the digestive enzymes of humans if they reach the small intestine unaltered (Belitz et al., 1982). Protease inhibitors were the cause of nutritional disorders, growth depression, and pancreatic hypertrophy and/or hyperplasia (Liener and Kakade, 1980) when rats, mice, or chickens were fed plant products containing high levels of protease inhibitors. In rodents and birds, growth depression is accompanied by enlargement of the pancreas due to cell hypertrophy and/or hyperplasia. Levels of trypsin inhibitor activity (TIA) in raw soybeans are high [50 trypsin inhibitor units (TIU) $mg^{-1}$ of dry matter (DM)] (Huisman 1993). Kakade et al. (1973) demonstrated that protease inhibitors are responsible for ~40% of the growth depression and pancreas enlargement observed in rats fed with soybean.

Pig-feeding studies have shown that grass pea can be used as a dietary component, but when levels exceed 20%, performance is reduced (Castell et al., 1994). Apparently, antinutritional factors, such as trypsin inhibitors and chymotrypsin inhibitors, rather than

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**Table 1. TIA of Field Pea Cultivars Grown in 10 Environments**

| cultivar | breeder | TIA (TIU mg$^{-1}$ of DM) mean | TIA (TIU mg$^{-1}$ of DM) estimate (sq root) |
|---|---|---|---|
| Danto | Prodana, Denmark | 2.22 | 1.49 |
| Fluo | Blondeau, France | 2.33 | 1.52 |
| Celeste | Nickerson, France | 2.39 | 1.54 |
| Carneval | Svalöf-Weibull, Sweden | 2.51 | 1.57 |
| Spring D | Danisco, Denmark | 2.52 | 1.58 |
| Montana | Cebeco, The Netherlands | 2.56 | 1.59 |
| Express | Svalöf-Weibull, Sweden | 2.76 | 1.64 |
| Miko | IHAR, Poland | 2.81 | 1.65 |
| Trump | Agriculture and Agri-Food Canada | 2.92 | 1.70 |
| Highlight | Svalöf-Weibull, Sweden | 2.95 | 1.70 |
| Bohatyr | Selgen, Czech Republic | 2.94 | 1.70 |
| Titan | Agriculture and Agri-Food Canada | 3.27 | 1.79 |
| AC Tamor | Agriculture and Agri-Food Canada | 3.31 | 1.80 |
| Richmond | Svalöf-Weibull, Sweden | 3.32 | 1.81 |
| Orb | Sharpes, UK | 3.35 | 1.82 |
| Patriot | Svalöf-Weibull, Sweden | 4.08 | 2.00 |
| Baroness | Sharpes, U.K. | 7.66 | 2.70 |
| mean | | 3.17 | 1.74 |
| SE of diff[b] | | | 0.09 |

[a] Estimate based on random effects model analysis of variance. Least significant difference between estimates at 5% level is 0.17 for preplanned comparisons (*t* test with 144 degrees of freedom). [b] Standard error of difference.

ODAP, limit the potential of grass pea as a feedstuff for swine.

The objectives of international grass pea breeding programs are to develop lines with reduced levels of the neurotoxin ODAP (Campbell, 1989) and selection for improved yield and early maturity (Ramanujan et al., 1980; Chandna et al., 1991). In comparison to research on ODAP, little information is available on the presence of TIA in grass pea. For field pea, several studies (Carrouée, 1996; Domoney and Welham, 1992; Leterme et al., 1990; Bacon et al., 1995a; Griffiths, 1984) have been reported on the presence of enzyme inhibitors, but few studies have examined the relative influence of genotype and environment on these factors.

To facilitate the breeding of Canadian cultivars for improved nutritional quality for export and domestic use, it was necessary to survey existing germplasm for levels of TIA. Thus, the objectives of this study were to determine whether genotype and environment have significant influences on TIA in field pea and grass pea and to determine whether TIA is correlated with seed protein content and seed yield.

## MATERIALS AND METHODS

Seed samples of 17 field pea cultivars (Table 1) commonly grown in western Canada were obtained from regional adaptation trials conducted at locations in Manitoba (Arborg, Dauphin, Minto, Rosebank, and Thornhill) during 1993 and 1994. For grass pea, seed samples of 9 breeding lines (Table 5) were obtained from Agriculture and Agri-Food Canada, Morden breeding program, which had been grown in preliminary tests in Morden and Portage la Prairie, MB, during 1993 and 1994. Field pea cultivars used included yellow and green cotyledon types; all cultivars had white seed coats. Samples of grass pea had a range of seed coat colors from white through dark brown. Whole grain samples for TIA analysis were ground to pass through an 80 mesh screen using a Thomas-Wiley intermediate mill equipped with stainless steel blades.

Moisture content of sample flours were determined according to the AOAC method (AOAC, 1990). Sample size was 2 g for field pea and grass pea. Protein content (N × 6.25) was determined according to the Kjeldahl method with a Tecator digester and a Kjeltec (System 1002) distillation unit (Tecator AB, Höganäs, Sweden). Sample size was 100 mg for field pea and grass pea.

TIA of sample flours of field pea and grass pea was determined on the basis of the method of Kakade et al. (1974). Sample size was 50 mg for field pea and 5 mg for grass pea; smaller samples were used for grass pea since TIA in grass pea was greater than in field pea. Extracts were prepared fresh daily as described by An et al. (1993). Field pea flour (50 mg) and grass pea flour (5 mg) were extracted on an orbital shaker (Lab-Line Instruments Inc.) with 20 mL of 0.009 M HCl for 1 h at room temperature. The extract was then centrifuged (Sorvall superspeed RC 2-B) at 10000*g* for 20 min. Five aliquots of between 0 and 2 mL (0, 0.5, 1, 1.5, and 2 mL) of supernatant solution were pipetted into test tubes. Sufficient distilled water was added to give a total volume of 2 mL. Two milliliters of trypsin solution (40 mg of bovine trypsin, Worthington Biochemical Corp., 3703, dissolved in 2000 mL of 0.001 M HCl) was added to each tube. To two other tubes was added 2 mL of sample supernatant (sample blank) or 2 mL of water (substrate blank). All of the tubes were placed in a water bath at 37 °C to equilibrate. After 10 min, 5 mL of substrate solution was added to each tube. Substrate solution consisted of 400 mg of *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) hydrochloride (Sigma, catalog no. B-4875), which was dissolved in 20 mL of dimethyl sulfoxide and allowed to stand for at least 30 min (1 mL aliquots of the resulting solution were then stored at −20 °C for future use). As required, 1 mL of BAPNA solution was diluted to 50 mL with freshly prepared 20 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.2, preheated to 37 °C. (The final substrate solution was then stored at 37 °C and discarded after 4 h.) After a further 10 min, the reaction was stopped by the addition to each tube of 1 mL of 30% v/v acetic acid. Two milliliters of trypsin solution was added to both the substrate and sample blanks. The $A_{410}$ values of the tube contents were measured in a spectrophotometer (Beckman DU 640B) using the substrate blank as reference. Several sets of tubes were analyzed at once, starting each, in sequence, at timed intervals with the addition of the enzyme solution.

All sample data values were corrected for sample blank as follows:

$$\text{corrected sample } (A_{410}) = \text{sample } (A_{410}) - [\text{sample blank } (A_{410}) \times \text{sample volume/2 mL}]$$

The corrected sample ($A_{410}$) values were plotted versus sample volume. TIA was calculated from the slope of the linear part of the plot as follows:

$$\text{TIA (TIU)/mg of sample} = \text{slope} \times \text{dilution factor}/[0.01\,6 \times (\text{total assay volume (mL)}/10) \times \text{sample concentration (mg mL}^{-1})]$$

TIUs were calculated from trypsin inhibition data in the range of 40−60% (Bacon et al., 1995b). One TIU is defined as a decrease in $A_{410}$ by 0.01 in 10 min using the large scale assay (Kakade et al., 1974; Stauffer, 1990). TIA was expressed in units of trypsin inhibited (TIU) per milligram of dry matter of the sample (Kakade et al., 1974). All chemicals used were of reagent grade. All chemical analyses were performed at least in duplicate.

Data were analyzed using a random effects model, and variance components and cultivar means were estimated using the SAS procedure Mixed (SAS Institute, 1992). Rather than estimate separate variance components for years and locations, the 2 years and 5 locations were treated as 10 environments. A square root transformation was applied to stabilize the

**Table 2. Effect of Environment on TIA of Field Pea Cultivars Grown in Manitoba**

| environment | TIA (TIU mg$^{-1}$ of DM) |
|---|---|
| 1. Minto, 1993 | 3.15 |
| 2. Thornhill, 1993 | 2.75 |
| 3. Dauphin, 1993 | 2.74 |
| 4. Rosebank, 1993 | 2.46 |
| 5. Arborg, 1993 | 2.88 |
| 6. Minto, 1994 | 3.07 |
| 7. Thornhill, 1994 | 3.94 |
| 8. Dauphin, 1994 | 3.20 |
| 9. Rosebank, 1994 | 4.38 |
| 10. Arborg, 1994 | 3.15 |
| mean | 3.17 |

**Table 3. Variance Components and Indicator of Heritability for TIA in Field Pea Cultivars Grown in 10 Environments[a]**

| covariance parameter | estimate | SE | probability | % of total |
|---|---|---|---|---|
| cultivar | 0.0823 | 0.0305 | 0.007 | 55.3 |
| environment | 0.0263 | 0.0135 | 0.052 | 17.7 |
| interaction | 0.0403 | 0.0047 | 0.000 | 27.1 |
| heritability indicator (%) | 67.1 | 8.6 | | |

[a] Square root transformed. Interaction includes error. Heritability indicator is cultivar divided by cultivar plus interaction. Asymptotic covariance between cultivar and interaction variance components is $-2.26 \times 10^{-6}$.

variance. Correlation analysis was conducted on the estimated cultivar means using SAS (SAS Institute, 1990).

## RESULTS AND DISCUSSION

**Field Pea.** Mean TIA levels in the 17 field pea cultivars tested differed significantly ($P < 0.01$) and ranged from 2.22 TIU mg$^{-1}$ of DM for Danto to 7.66 TIU mg$^{-1}$ of DM for Baroness (Table 1). Baroness had significantly higher levels of TIA than all other cultivars. Patriot had significantly higher levels of TIA than all other cultivars except Baroness. The level of TIA in a cultivar did not appear to be associated with the breeding program that developed it. For instance, the Sharpes cultivar Baroness had the highest level, whereas another Sharpes cultivar, Orb, had an intermediate level of TIA. Environments did not differ greatly in mean TIA levels (Table 2). Analysis of variance for TIA in field pea showed that cultivar (55.3% of total variation) had a larger relative contribution than environment (17.7% of total variation), and the heritability indicator for TIA was relatively high (67.1%) (Table 3).

Gatel and Grosjean (1990), summarizing the literature on this topic, reported that TIA in field pea ranged from 0.52 to 12.5 TIU mg$^{-1}$ of DM. Carrouée (1996) reported that TIA levels of Baroness, Express, Montana, and Carneval were 11.1, 3.7, 3.5, and 2.9 TIU mg$^{-1}$ of DM (unpublished data). Slinkard and Tyler (University of Saskatchewan, unpublished data) reported that TIA levels of Baroness and Express were 9.6 and 4.4 TIU mg$^{-1}$ of DM. Domoney and Welham (1992) reported that TIA levels of five field pea genotypes were 3.0−16.3 TIU mg$^{-1}$ of DM. Leterme et al. (1990) determined TIA levels of 33 European spring field pea varieties, which ranged from 1.71 to 8.40 TIU mg$^{-1}$ of DM. TIA level of Danto was 1.94 TIU mg$^{-1}$ of DM. TIA levels were determined in seeds from 11 field pea cultivars grown at 7 European locations during the 1990 season by Bacon et al. (1995a). Mean values of TIA were 0.93−2.55 TIU mg$^{-1}$ of DM. Griffiths (1984) reported that TIA levels ranged from 0.15 to 4.62 TIU mg$^{-1}$ of DM in 18 field pea cultivars. Thus, our data showed that levels of TIA in field pea cultivars grown in western Canada were similar to those previously reported in the literature.

**Table 4. Correlation Coefficients for TIA, Seed Yield, and Seed Protein Content in Field Pea between Cultivars (Mean of 10 Environments, $n = 17$)[a]**

| | seed yield | seed protein content |
|---|---|---|
| TIA | −0.171 | 0.244 |
| seed yield | | −0.778[b] |

[a] All variables are square root transformed. [b] $P < 0.01$.

**Table 5. TIA in Grass Pea Breeding Lines Grown in Four Environments**

| | TIA (TIU mg$^{-1}$ of DM) | |
|---|---|---|
| line | mean | estimate (sq root) |
| L880294 | 30.79 | 5.23 |
| L880388 | 28.35 | 5.22 |
| L900431 | 28.74 | 5.22 |
| LS89026 | 23.78 | 5.21 |
| LS89110 | 26.53 | 5.22 |
| LS89125 | 29.38 | 5.23 |
| LS90040 | 24.36 | 5.21 |
| LS90043 | 27.62 | 5.22 |
| LS90045 | 28.07 | 5.22 |
| mean | 27.51 | 5.22 |

Cultivar mean levels of TIA were not correlated with seed yield or protein content in field pea, although there was a strong negative correlation between yield and protein content (Table 4).

Field pea is an underutilized resource in animal feeding. Field pea meal is highly palatable to pigs and recommended at levels of 25−30% of the ration (Bell and Wilson, 1970; Vogt, 1983; Gatel and Grosjean, 1990). Field pea cultivars were much lower in TIA (with mean level of 3.17 TIU mg$^{-1}$ of DM) than raw soybeans (50 TIU mg$^{-1}$ of DM) and less than heat-treated soybean meal (5 TIU mg$^{-1}$ of DM) (Huisman, 1993). The cultivars Baroness and Patriot had relatively high levels of TIA and should be avoided in the feed industry and in breeding for low TIA. Thus, field pea is an excellent feed ingredient due to its high protein content, high dietary energy levels, low levels of TIA, and low levels of condensed tannins and total phenolics (Wang et al., 1998). Feed use of field pea in western Canada can be expanded.

**Grass Pea.** TIA in grass pea did not differ among lines (Table 5) or environments ($P > 0.05$). The mean value of TIA in grass pea was 27.51 TIU mg$^{-1}$ of DM. Roy and Bhat (1975) reported that TIA levels in 10 grass pea cultivars ranged from 11.1 to 28.5 TIU mg$^{-1}$ of protein. Aletor et al. (1994) reported that TIA levels in 36 grass pea lines maintained in the germplasm collection at International Center for Agricultural Research in the Dry Areas (ICARDA) were 13.88−23.22 g kg$^{-1}$ of DM. Deshpande and Campbell (1992) reported that the range for TIA in 100 lines of grass pea germplasm was 133−174 TIU mg$^{-1}$ of DM. However, their TIA analysis and calculation methods (Deshpande et al., 1982) differed from ours.

Cultivar mean levels of TIA in grass pea were not correlated with seed yield ($r = 0.244$) or seed protein content ($r = 0.177$). There was no correlation between seed yield and seed protein content ($r = 0.653$).

In summary, field pea cultivars grown in western Canada had low levels of TIA, except for Baroness and

Patriot. The levels of TIA in field pea were mainly dependent on genotype; the effect of environment was relatively small. There was no correlation between the levels of TIA and seed yield in field pea. Field pea cultivars with low TIA levels should be used as parents to breed for low TIA cultivars. Field pea cultivars with relatively high TIA levels, such as Baroness and Patriot, should be avoided. TIAs in grass pea did not differ among cultivars tested. Mean levels of TIA in grass pea lines analyzed were 8.7 times greater than those of the field pea cultivars tested. The TIA levels in grass pea are of more concern in nutrition than those in field pea. Surveying germplasm collections of grass pea to identify accessions with low TIA is necessary to breed low-TIA cultivars and to improve the value of grass pea as a feed ingredient.

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