Canola Agronomic

Research Program

Final Report of Project

1. Project Title

A Biochemical test for Low Seedling Vigour in Canola

3. Status, Commencement & Duration of Project

Commencement Date: April, 2000

Duration of entire project in years, 3.5 yr

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6. Brief Description (30 words)

Objectives are: 1) to discover the biochemical causes of low seedling vigour in canola and 2) to develop a vigour test based on the new knowledge.

ABSTRACT

The biochemistry of canola seed/seedling vigour has been studied and the development of a new vigour assay based on seed metabolism is nearing completion. Study of sulphur metabolism revealed an unexpected requirement for sulphur and other nutrients in very young seedlings (age 1-5 d). Although sulphur metabolites, per se, were not found to be highly correlated with vigour, high vigour seed had higher concentrations of sulphur than low-vigour seed. A volatile indicator of fatty acid peroxidation, pentane, was shown to be emitted by seeds, but was not correlated with vigour. During the studies of fatty acid peroxidation, three other volatile compounds emitted by canola seed were found to be strongly correlated with vigour. Two of the compounds were identified as ethyl alcohol and acetaldehyde, which are an end-product and an intermediate of sugar fermentation. Ethyl alcohol was by far the most abundant

compound emitted by low-vigour seed. Study of a large seed sample set, consisting of 151 seed lots and including more than 50 non-hybrid and hybrid varieties and 19 seed treatment formulations, revealed a high correlation ($r = 0.81$) between seed vigour and ethanol emissions. Results indicated that seed treatment, hybridization and genetic and mutagenic modification had minimal effect on the correlation between vigour and ethanol emissions. Two vigour assays based on ethyl alcohol emission by canola seed are being developed. They are a colour test that is designed for on-farm use and an instrumental technique that will be suitable for use by agri-business.

INTRODUCTION

Seed germination, seedling emergence, and crop establishment are important aspects of agricultural and horticultural production, and are the main components of seed/seedling vigour. These factors are related to early growth of the crop, and may be related to resistance to early-season stresses and final yield. A major concern of growers is that deterioration of some seed lots, leading to loss of vigour, may be undetected before planting. Current vigour tests vary in reliability and in their applicability to individual crops. Canola is one crop for which a reliable vigour test is not available. Poor vigour canola may be particularly susceptible to early season stresses such as cold, insects and weeds. Since vigour changes with time, the vigour of seed should be tested shortly before planting.

Relatively little research has been conducted on seed or seedling vigour in canola. Most vigour studies have been performed with rice, cotton, soybeans and wheat. Little or no yield advantage from planting large versus small seeds was observed in earlier studies with rape (Kondra 1977; Major 1977). However, individual seeds planted far enough apart to minimise competition among plants lead to higher yields from large seeds compared to small seeds of rape (Ahmed and Zuberi 1973). A comparison of emergence of several crops over a range of soil temperatures and moisture revealed that percentage emergence of winter canola was considerably less than that of downy brome, wheat and rye at low temperatures, which indicated the relative intolerance of canola to low temperatures during germination and emergence (Blackshaw 1991). Recent work with canola showed that low temperature germination potential varied considerably among seed lots due to variation in the ability of the seed to utilise stored reserves (Johnson-Flanagan 1993).

In current work at the Saskatoon Research Centre, one of the co-investigators, R.H. Elliott, is evaluating a variety of seed and seedling vigour tests for their ability to distinguish between high- and low-vigour canola seed lots.

The objectives of the study described herein were to investigate the metabolism of canola seed and seedlings and, if possible, use the results of the investigation to develop a new vigour test for canola. The following report is divided into three sections, one for each of the major lines of investigation followed in the research: sulphur metabolism; fatty acid oxidation; and carbohydrate fermentation, which led to the development of ethanol-based vigour assays.

SULPHUR METABOLISM

Background

Seedling vigour appears to be related to the ability of young seedlings to tolerate various stresses during early growth. Sulphur metabolism, and, in particular, a sulphur metabolite called glutathione (GSH), has been identified as playing a central role in plant tolerance to a number of stresses (Noctor et al. 1998;May et al. 1998). Glutathione is involved in herbicide tolerance, disease resistance, heavy metal tolerance, cold tolerance and resistance to oxidative damage. All of these stresses enhance glutathione synthesis. Glutathione or GSH derivatives conjugate herbicide and chelate heavy metals leading to their detoxification. Also, damaging oxygen species generated during disease stress and oxidation of unsaturated oils are inactivated through a series of reactions that involve GSH.

Techniques and Instrumentation

A hydroponics system was designed and set up for the investigation of the metabolism of very young seedlings of canola. Pre-existing high performance liquid chromatography techniques for the determination of sulphur metabolites were adapted for analysis of canola root tissue. The study of several aspects of sulphur metabolism were undertaken, including work on stresses expected to affect seed vigour. These stresses were 1) cold stress, achieved by exposure to various day/night temperature regimes and 2) hypoxia stress, imposed by bubbling N_2 and air in various proportions in the hydroponics solution.

Results

Several low-molecular weight thiol compounds were identified by high performance liquid chromatography in canola seedling roots. These were cysteine, glutathione, a five-peptide phytochelatin (called PC2) and a seven-peptide phytochelatin (called PC3). Because glutathione plays a central role in sulphur metabolism as well as in stress response, it was the main metabolite of interest. Cysteine is an amino acid precursor of glutathione and the phytochelatins (PCs) are synthesised from glutathione.

Analysis of roots of 6- or 8-d old seedlings revealed no difference in levels of sulphur metabolites between high- and low-vigour seed lots when the seedlings were raised under ideal conditions (22-27 °C day; 17 °C night). When exposed to a 48-h cold treatment (12-17 °C day; 2-5 °C night) prior to harvest, cysteine and glutathione increased in the roots of all plants. However, there was no significant difference in response between high- and low-vigour seed lots (data not shown).

Hypoxia also was found to significantly affect the concentration of glutathione in canola roots. As the percentage saturation of oxygen in the hydroponics solution was decreased from about 25 to 5%, the concentration of glutathione nearly doubled in response to the developing anoxia in the root tissue. Unexpectedly, we observed that this effect only occurred in the absence of supplemental sulphur. In the presence of supplemental sulphur, glutathione concentration was high and remained relatively constant from 5 to 100% oxygen saturation, which suggested that sulphur status of the seed may be a determining factor in glutathione stress response. The difference in glutathione response to hypoxia between high- and low- vigour seed lots was not studied.

Because of the unexpected sulphur response, we investigated the effect of sulphur and a complete nutrient solution on the growth of canola seedlings. We observed a substantial effect of the supplemental nutrients on early growth (0-5 d). Although it is generally believed that seeds contain sufficient nutrients for the first 7-10 days of growth, this is apparently not the case for canola. We found that 2-3 times the shoot biomass was produced by canola seedlings when provided with the nutrients. Figure 1 shows the differences in growth observed with high- and low-vigour lots of AC Excel grown for five days (from the start of imbibition) with or without available nutrients. Elemental analysis of canola seed from 26 high- and low-vigour lots showed no significant difference ($P > 0.05$, paired t-tests) between lots for calcium, magnesium, phosphorous, potassium, copper, iron, manganese and zinc (Table 1). Small but statistically significant differences in nitrogen and sulphur concentrations between the high and low-vigour lots were found. Nitrogen and sulphur are both components of protein, and the observed differences may reflect an association of high protein with high vigour. It is also possible that sulphur may be acting independently of protein in the form of glutathione and related compounds. The results of the elemental analysis indicated that elemental composition alone would not be a basis for a vigour test. However, it is apparent from the nutrition work to date that there may be a potential for increasing the vigour of canola seed by controlled supplementation of the seed with nutrients. Because such an objective was not within the scope of the current project, other funding was obtained to pursue the investigation of nutritional supplements.

LIPID PEROXIDATION

Background

The relationship between deterioration of seed quality and lipid peroxidation was reviewed by Wilson and McDonald (1986a). Since volatile aldehydes are produced during lipid peroxidation, an assay involving the measurement of volatile aldehydes from germinating soybean seed was proposed as a test of seed vigour (Wilson and McDonald 1986b). In more recent work on seed deterioration in soybeans (Trawatha et al. 1995), it was concluded that free fatty acids might have contributed to seed deterioration by disrupting membranes and/or by toxicity of subsequent peroxidation products. Even small amounts of lipid peroxidation byproducts, called lipid hydroperoxides, are toxic to living organisms.

A problem with the measurement of volatile aldehydes is that these compounds are readily metabolised by microorganisms found with the seed (Harman et al. 1982). Although apparently not studied as indicators of lipid peroxidation in plants, several low molecular weight hydrocarbons have been studied as peroxidation endproducts in animals. The expiration of ethane and pentane by animals has been successfully used as a measure of lipid peroxidation in rats subjected to stresses (Sword et al. 1991). Ethane is an endproduct of linolenic acid peroxidation and pentane is an endproduct of linoleic acid peroxidation. These hydrocarbons are more stable than aldehydes and less likely to be used as substrates by micro-organisms. Consequently, they were chosen for the investigation of lipid peroxidation in canola.

Techniques and Instrumentation

Volatile compounds found in the head space gas above moist seed were analysed by gas

chromatography. Seed was weighed into vials of various sizes and water was added to the seed to make it up to 20 % moisture. The vials were sealed and incubated for 24 h at room temperature. Volatile compounds in the head space gas were pre-concentrated and separated from water vapour by means of automated solid phase micro-extraction. A 30-metre capillary column with a non-polar immobile phase was used to separate the analytes, which were detected and quantified by means of a flame ionisation detector.

The separated analytes appeared as peaks in the gas chromatography output (Figure 2). The pentane peak was identified by introducing purified pentane into the seed head space gas and also into empty vials in order to match the retention time of pentane with that of peaks in the chromatograms.

The relationship between fatty acid peroxidation and seed vigour was studied with a set of 13 pairs of untreated canola (*Brassica napus*) seed samples (sample set 1). Each pair consisted of a high-vigour and a low-vigour sample of the same variety. The seed was from a project of one of the co-investigators, R.H. Elliott. Sample set 1 was obtained as a blind sample set with respect to variety. The emission of pentane also was evaluated in a second set of 40 unpaired samples from R.H. Elliott (sample set 2, *Brassica napus*) and a set of 111 unpaired seed samples (*Brassica napus* and *Brassica rapa*) from growers and members of the seed industry (sample set 3). Sample set 2 was obtained as a blind sample set with respect to vigour and variety. Fourteen of the samples were treated with a fungicide and 26 were untreated. Sample set 3 included 51 varieties and hybrids and 19 different fungicide and insecticide treatment formulations and the vigour of all samples was unknown. Sample set 3 consisted of varieties and seed treatments in recent use in Western Canada. The vigour of the seed in sample set 1 was estimated by several methods as part of another project (R.H. Elliott).

The vigour of seed in sample sets 1, 2 and 3 was determined by growing seedlings in hydroponics. The hydroponics bioassay consisted of placing 100 canola seeds on a stainless steel mesh suspended about 1 cm above an aerated complete nutrient solution in 4-litre plastic containers. Bubbling in the nutrient solution created a mist that wetted the seeds, yielding suitable conditions for germination and growth. The seed was incubated for 5 d at 22 °C during 16-h daily light periods (relatively low light level) and 17 °C during dark periods. Humidity was kept high (about 90 %) by using translucent plastic covers with ventilation holes. The vigour determinations of sample set 1 seed included daily counting of germinated seeds, daily counting of seedlings that had undergone hypocotyl (the stem between the root/shoot junction and the cotyledons) elongation (greater than 7 mm), and fresh weight of shoots and dry weight of roots at 5 d. A function combining the four factors was used to represent vigour in sample set 1. Total fresh weight (roots and shoots) was used to represent vigour in sample sets 2 and 3.

Relationships between variables were investigated by linear regression (SAS Institute 1990).

Results

In preliminary investigation, only very small amounts of pentane and other gasses were emitted by air-dried canola seed. The gasses were barely detectable by gas chromatography. However, it was soon discovered that the addition of moisture to the seed greatly enhanced the evolution of gasses and thereby permitted further investigation.

The evolution of pentane from canola seed was found to be quite variable (sample set 1, Figure 3). In some seed lots elevated pentane evolution was associated with low vigour,

however, the effect was not consistent across all seed lots. Although work with sample set 1 (Figure 3) suggested a weak relationship between seed vigour and lipid peroxidation, as more data was accumulated it became clear that no significant correlation existed (sample sets 2 and 3, Figure 4). Thus, it appears that lipid peroxidation is not an important factor in vigour of canola seed in Western Canada. This conclusion contrasts with that of researchers studying soybean vigour (Wilson and McDonald 1986b, Trawatha et al. 1995), who concluded that lipid peroxidation was a significant factor. Their conclusion was based on aldehyde production by seed. However, acetaldehyde, an important intermediate in carbohydrate fermentation, would also be measured with their techniques along with the group of aldehydes produced by lipid peroxidation. It appears that the earlier workers, looking for production of aldehydes by low-vigour seed as an indicator of lipid peroxidation, did not consider the possibility that the aldehyde they found was due to another process, namely an increase in carbohydrate fermentation.

CARBOHYDRATE FERMENTATION

Introduction

During the investigation of lipid peroxidation, it was discovered that low-vigour seed appeared to be emitting large quantities of an unidentified volatile compound that was visible on the chromatograms. This compound was soon identified as ethanol by addition of an internal standard (the same technique used for identifying pentane). Preliminary investigation indicated that measurement of ethanol emissions might be useful for distinguishing between high- and low-vigour canola seed. Consequently, resources were directed toward this promising new development.

Background

Most organisms including plants have the ability to derive metabolic energy from an anaerobic process called fermentation. Fermentation involves the conversion of simple carbohydrates, mainly glucose, into several possible intermediates and endproducts including an aldehyde, called acetaldehyde, and ethanol.

Gorecki et al. (1985) examined volatile exudates from germinating pea (*Pisum savitum*) and showed that as storage time increased, viability decreased. The quantities of ethanol and acetaldehyde produced by the germinating seeds was somewhat proportional to the age of the seed, increasing as seed age increased. They noted that determinations of acetaldehyde and ethanol in the space over germinating seeds by means of gas chromatography might be a useful seed vigour test. Gorecki et al. (1992) also analysed volatile organics produced by starchy pea seeds and fatty cocklebur (*Xanthium pennsylvanicum*) seeds following water imbibition. Aged seeds produced larger amounts of ethanol and acetaldehyde, although in cocklebur seeds, acetaldehyde was the preponderant end product.

Muskmelon (cantaloupe, *Cucumis melo*) seeds were aged artificially for up to 12 d at 45 °C and 100 % relative humidity. Artificial ageing reduced their ability to germinate and increased the production of ethanol and acetaldehyde during imbibition. The authors suggested that ethanol production in the first hours of imbibition might be used as a method to predict germination in muskmelon seed (Pesis and Ng 1986).

Naturally or artificially aged soybean (*Glycine max*) seed had higher ethanol and acetaldehyde concentrations in seed tissue than did un-aged seed, although the difference between aged and un-aged seed varied greatly with hours of imbibition, rate of water uptake and temperature (Woodstock and Taylorson 1981).

Techniques and Instrumentation

Analytical techniques were similar to those described for lipid peroxidation. Peaks in gas chromatography output, however, were identified by two procedures. Firstly, purified known compounds (internal standards) were introduced into the seed head space gas and empty vials as described above. Secondly, the gaseous output from the gas chromatography column was introduced into a mass spectrometer and information on the masses of eluted compounds and their degradation products was obtained. Compounds were identified by correlation of the measured masses with those of known compounds. Mass spectrometric analysis was performed by G. Eigendorf, University of British Columbia. Not all compounds of interest could be identified.

The effect of seed moisture was investigated by measuring 1-g samples of high- and low-vigour canola seed with water added to adjust the seed moisture from 5 to 50 %. Low-molecular weight volatile compounds in the head space gas were determined after 24 ± 2.5 h incubation at room temperature.

The effect of incubation time at room temperature after addition of water to seed in sealed vials was investigated.

Sample sets 1, 2 and 3 were used in the carbohydrate fermentation studies as well as the lipid peroxidation studies. Vigour was determined by the hydroponics techniques as described above. Volatile compounds emitted from the moist seed were determined by gas chromatography.

Relationships between variables were studied by linear regression (SAS Institute 1990). Regression coefficients (slopes) and correlation coefficients were compared as described by Zar (1984).

Results

As described below, low-molecular weight gases found in the head space of moist canola seed were identified by gas chromatography and mass spectrometry. Also, the analysis of sample sets 1, 2 and 3 indicated the potential of using ethanol emissions as the basis of a vigour assay.

Several peaks in the gas chromatograms were identified. Unknown A was identified as acetaldehyde by the addition of an internal standard and confirmed by mass spectrometry. Unknown D was identified as dimethyl sulphide by mass spectrometry and confirmed by addition of an internal standard in gas chromatography. The identity of the ethanol peak was confirmed by mass spectrometry. Unfortunately, though, no further compounds could be identified by mass spectrometry. Further work with internal standards and gas chromatography indicated that unknown C could be any combination of several compounds, all of which are known metabolic intermediates or endproducts: pyruvic acid, acetone and acetoin. These compounds can not be resolved by the current gas chromatographic procedure. The failure to identify unknown C by mass spectrometry may be at least partially because it could be a mixture of compounds. A series of aldehydes were added as internal standards in gas chromatography, but none had retention times equivalent to unknown peaks. The aldehydes tested were

valeraldehyde, hexanal, trans-2-hexenal, heptaldehyde, trans-2-nonenal and nonyl aldehyde. In addition, the following compounds were tested as internal standards and not found to be one of the unknowns: acetic acid, propionic acid, butyric acid, lactic acid, hexane and octane. Although unknowns E and F have not been identified, we can state that they are not any of the above-mentioned compounds. Both propionic acid and butyric acid, however, had retention times similar to unknown F. In future work, it could be useful to modify the gas chromatography techniques so that the potential mixture in unknown C can be resolved. If it is a mixture, it is possible that one or more of the compounds could be an indicator of vigour.

Typical gas chromatograms obtained from the analysis of high- and low-vigour seed lots are shown in Figure 2. The ethanol peak appearing at approximately 5.5 min was much larger in most of the low-vigour seed samples compared to the high-vigour seed samples.

Moisture percentage had a pronounced effect on the evolution of ethanol from low-vigour canola seed and only a minor effect on the high-vigour seed (Figure 5). Similar results were obtained for acetaldehyde and Unknown E (not shown). From these results it might appear that a moisture content of about 35 % might be ideal for the head space gas analysis. However, variation in analytical results increased with higher moisture percentages. To test the significance of this effect on the ability to distinguish between high- and low-vigour samples, sample set 2 was tested at 20 and 35 % moisture. Correlation coefficients between the hydroponics vigour test and ethanol, acetaldehyde and Unknown E emissions were -0.78, -0.72 and -0.73, respectively, for the seed at 20 % moisture. However, at 35 % moisture correlation coefficients were –0.60, -0.57 and –0.62, respectively. The results indicated that the ability to resolve high- and low-vigour seed based on ethanol, acetaldehyde or Unknown E determinations was better at 20 % moisture compared to 35 % moisture. Thus, control of the moisture percentage of the seed is important when using ethanol determinations to estimate seed vigour.

The concentration of ethanol in the head space gas of canola seed at 20 % moisture increased in a linear manner during a test period of 16 to 40 h (Figure 6). As a result measurements of ethanol and other gasses in head space gas were normally performed during a 5-h window, that is 24 ± 2.5 h after adding water to the seed, although a 10-h window (24 ± 5 h) was used on occasion. Some variation in time was necessary for practical considerations with respect to gas chromatography analysis.

The source of ethanol emitted from canola seed could be the seed, itself, microbes on the seed, or both. Samples of 10 lots of high- and low-vigour canola seed were surface-sterilised by exposure to a 1:5 dilution of household bleach for 10 min. Ethanol, pentane and unknown volatiles B and F were largely unaffected by surface sterilisation, although a reduction in ethanol of two seed lots after surface sterilisation may indicate that a component of the gas production could be microbial. It should be noted, though, that the high-vigour seed lots produced negligible ethanol. If significant ethanol production were derived from microbial activity, then one might have expected some ethanol production from high-vigour seed. On the other hand, dimethyl sulphide was nearly eliminated by surface sterilisation in all seed lots. Dimethyl sulphide is a well-known metabolite of the brassica family. Dimethyl sulphide is readily oxidised and since bleach is a powerful oxidising agent, residual bleach on the seed may have been strong enough to oxidise any dimethyl sulphide present. In general, this study indicated that ethanol produced by moist canola seed is mostly, if not all, generated by the metabolism of the seed.

Since the original vigour designations of sample set 1 provided by R.H. Elliott were estimates only, all 26 seed samples were subjected to the hydroponics vigour bioassay described above. It was found that the high-vigour seed samples of different varieties and hybrids of canola differed somewhat in their vigour, apparently due to genetic variation. In the present study, we are not concerned with measuring genetic variations in vigour among varieties or hybrids, but, instead, we wish to identify those seed lots that have lost vigour. Thus, in order to compare the low-vigour seed lots to the high-vigour lots, the high-vigour seed lots (as originally estimated by R.H. Elliott) were assigned a vigour status of 100. Within each pair, the vigour of the low-vigour lot was expressed as a percentage of its corresponding high-vigour lot.

Analysis of volatiles in headspace gas of each seed sample (after incubation at 20 % moisture for 24 hours at room temperature) was determined in triplicate by gas chromatography. Correlation coefficients between the bioassay vigour rating and measurements of ethanol, acetaldehyde and unknown E were found to be –0.74, -0.78 and –0.78 ($P < 0.05$), respectively. Acetaldehyde is an intermediate found in the conversion of glucose to ethanol. The relatively high correlation coefficient for unknown E suggests that it, too, may be an intermediate or endproduct in the fermentation of glucose. Tests of several possible metabolites, however, were unsuccessful as described above. The ethanol, acetaldehyde and unknown E found in headspace gas of each canola seed lot is compared with its bioassay vigour value in Table 2.

Because sample set 1 was composed of paired high- and low-vigour seed lots within varieties, it was suitable for estimating the reliability of using ethanol emissions for determining vigour. Results of ethanol analysis in this and other sample sets indicates that ethanol peak area counts (as determined by the gas chromatography procedure) up to 750,000 are consistent with high vigour. Thus, when estimating vigour using the ethanol assay, one would reject all seed with peak area counts > 750,000 as poor vigour. When that criterion was applied to sample set 1, all seed samples with less than 70 % vigour were rejected (area > 750,000 counts) and all samples with greater than 80 % vigour were accepted (area < 750,000 counts) (Figure 7). These results are consistent with dividing vigour into three categories: high (> 80 %), marginal (70 – 80 %) and low (<70 %) (shaded areas in Figure 7). Although seed ethanol emissions were effective for identifying high (> 80 %) and low (< 70 %) vigour in sample set 1, results were not definitive for the marginal region (70 – 80 % vigour). Results for acetaldehyde and unknown E for the same samples are shown in Figures 8 and 9.

Sample sets 2 and 3 were too diverse with respect to genetics and seed treatments to be organised into high/low vigour pairs as was done in sample set 1. Duplicate 0.5-g subsamples of seed were made up to 20 % moisture in sealed vials and, after 24 ± 2.5 h at room temperature, were subjected to head space gas analysis by gas chromatography. Results for ethanol determinations are shown in Figure 10. Because the data cannot be presented in high/low vigour pairs, variation in the horizontal axis includes genetic variation as well as vigour loss. We cannot distinguish between these two sources of variation in unpaired samples. Correlations of all measured low molecular weight components with results of the hydroponics vigour bioassay are shown in Table 3. The results of sample sets 1, 2 and 3 show that ethanol is a good indicator of seed vigour in a diverse collection of canola seed samples.

Sample sets 2 and 3 can be subdivided into several categories including untreated (bare) and treated lots; treatment with fungicide and treatment with fungicide plus insecticide; hybrid and non-hybrid varieties; and conventional, genetically modified and mutagenically derived varieties (Table 4). When the 151 seed lots were divided into untreated (75 lots, Figure 11) and

treated (76 lots, Figure 12) categories, correlation coefficients were -0.85 and -0.73, respectively. Although the difference between the two coefficients was not statistically significant ($P > 0.05$), there may be a trend to poorer correlations with treated seed compared to untreated seed. On the other hand, a higher proportion of treated seed lots were low vigour, and that might have influenced the difference in correlation coefficients. When the treated seed lots were further divided into those treated with fungicides only (44 lots, Figure 13) and those treated with fungicides and insecticides (22 lots, Figure 14), correlation coefficients of -0.76 and –0.71 were obtained. The insecticide in most of the 22 lots was lindane. Since lindane is no longer in use, it will be necessary to test insecticides that have recently replaced it. It is possible that chemicals used as carriers or adjuvants in seed treatment might influence correlation coefficients and should be further investigated. (Note that the sum of the treated lots is only 66 whereas there were a total of 76 treated lots in Sample sets 2 and 3. The discrepancy arises because the seed treatment for 10 lots was not identified.)

Division of sample sets 2 & 3 into hybrid (23 lots, Figure 15) and non-hybrid (123 lots, not shown) varieties yielded correlation coefficients of –0.84 and –0.80, respectively (Table 4).

Conventional varieties (37 lots) yielded a correlation coefficient of –0.86, whereas all genetically modified varieties (54 lots) yielded a correlation coefficient of –0.78 (Table 4). Two of the genetically modified genotypes were represented by more than 10 lots and they were included in Table 4. Nineteen lots with a mutagenically derived herbicide tolerance yielded a correlation coefficient of –0.77.

Four lots with a water resistant seed coating and five lots with a porous seed coating also were tested. The water resistant seed coating interfered with the determination of vigour by the hydroponics bioassay and, therefore, could not be evaluated. Ethanol emissions were consistent with vigour (bioassay) for lots with the porous seed coating; however, more samples need to be tested to determine the relationship reliably.

Twelve hybrid and non-hybrid varieties in samples sets 2 and 3 were represented by multiple samples, one or more of which appeared to be at least as vigourous as the high-vigour check (AC Excel). There were a total of 53 samples for the 12 varieties. Variety-to-variety differences in growth rate were eliminated for these varieties by expressing the hydroponics biomass results as a percentage of the highest biomass within each variety. Once the data was converted in this way, it was evaluated with respect to reliability of estimating vigour from ethanol emissions (Figure 16). As was adopted previously for sample set 1, a cut-off ethanol peak area of 750,000 counts was used. Rejecting all samples above 750,000 area counts did not reject any with growth rate greater than 80 % of the high-vigour check. However, accepting those with less than 750,000 area counts resulted in accepting 5 seed lots between 50 and 70 % vigour. There were 16 results falling in the marginal range between 70 and 80 % vigour that were mostly accepted. All samples with less than 50 % vigour were rejected.

It is generally agreed that a laboratory or on-farm vigour assay must be consistent with field vigour determinations. A subset of sample set 2, consisting of 17 samples, was provided by one of the co-investigators, R.H. Elliott, who evaluated the samples for several field and laboratory vigour criteria. The laboratory vigour tests included several cold vigour tests and variations on the standard germination test. In general, good correlations were found between the results of tests performed by Dr. Elliott and the hydroponics assay and ethanol emissions (Table 5). Although the correlations were quite good with the 4-d germination test, they were

weakest (but still statistically significant; $P < 0.05$) with the standard 7-d germination test. Although a 7-d germination test is a measure of total germination percentage, the 4-d test is at least in part a measure of rate of germination. Our results from sample set 1 have shown that vigour as determined by 5-d hydroponics biomass is well correlated with rate of germination as one might expect.

In summary, studies of 177 seed samples (sample sets 1, 2 and 3) indicated that ethanol emission from moist seed is well correlated with hydroponics biomass production as well as field biomass production. Furthermore, ethanol emissions were not significantly influenced by a number of factors including seed treatment, hybridization, genetic modifications and mutagenic modification. Thus, it appears that measurement of ethanol emissions from moist canola seed may serve as the basis for practical vigour assays.

ON-FARM CANOLA SEED VIGOUR ASSAY

Introduction

The potential for an on-farm vigour assay was realised when it was discovered that a substantial amount of ethanol was given off by low-vigour canola seed. A trace of ethanol can even be detected by smell. A number of objectives have been, or are being, addressed during development of the on-farm assay. Firstly, a simple colour change reaction used in early breathalyser technology was adapted for use with canola. This required the testing of 17 modifications of the colour reagents until a suitable reaction time and colour intensity combination was achieved. Secondly, shelf life of the kit is being evaluated. Achieving an acceptable shelf life for the kit is a very important if the kit is to be in general use. Thirdly, the suitability of the assay for the ambient temperatures and seed moistures found on the farm was evaluated. It was assumed that an ambient temperature range of 19 – 27 °C (66 – 81 °F) and a seed moisture range of 4.5 to 10.5 % would be required. Fourthly, a number of configurations for an on-farm assay kit were tested. Finally, the reliability and accuracy of the on-farm assay is being tested against laboratory and field vigour measurements.

The current kit configuration includes a 2-oz clear glass bottle with chemicals dried on the interior surface. The bottle cap incorporates a clear FEP (fluorinated ethylene propylene) window, a glass fibre disc with dried colour reagents that are visible through the window, and two perforated Teflon discs. The assay is run by adding 2 g of canola seed (measured volumetrically in a small test tube) to the bottle, followed by 0.5 ml of water (measured with a small syringe). After capping, the bottle must be shaken vigorously for a about 10 s to dissolve the chemicals and distribute the moisture throughout the seed. Then it is set aside at room temperature and out of sunlight for 24-30 h. Low vigour seed is indicated by the development of a blue colour in the reagent disc; high vigour seed by a yellow colour. High and low-vigour seed samples analysed with the current version of the on-farm assay are shown in Figure 17.

Shelf life

A number of details in the kit construction must be considered in order to develop an assay kit that will be stable during extended storage. The colour reagents are very corrosive. They were found to react with a large variety of plastics except for fluorocarbon polymers such as Teflon

and related compositions. Also, the colour reagents are dry when incorporated into the kit, but they are very hygroscopic and eventually will absorb enough moisture from the atmosphere to become liquid. When dry, the colour reagents are sensitive to light. After about two week's exposure on a bench to normal fluorescent light, the reagents were found to discolour slightly. The discolouration was associated with a significant change in the colour response during the assay. It was found that containers constructed of polypropylene and polyethylene absorbed a significant amount of the ethanol produced by canola seed and, therefore, could not be used in the assay kits. Pressure can increase in the assay containers due to the emission of gasses from the seed and, therefore, it is important that the containers seal well. We have concluded that 1) the kit must be constructed so that only glass, Teflon and related plastics are in contact with the reagents and the gases emitted from the canola seed; 2) the kit must be protected from light and moisture during storage and 3) the assay container must seal well.

Shelf life tests have been performed by exposing assay kits to 70 °C in air and in water for up to 10 d, and then using the kits to run assays with seed of known vigour. Exposure to 70 °C for three days in air is used by industry as an approximation of two year's shelf life for commercial colour assays with similar chemistry (personal communication, marketing manager, Draeger Safety AG & Co., L⟨beck, Germany). Successful 10-d shelf-life tests at 70 °C in both air and water have been performed with a version of the on-farm assay kit. However, modifications made recently to the kit have not yet been evaluated for shelf life.

On-farm Assay Tolerance to Ambient Temperature and Seed Moisture Variation

One problem with laboratory seed vigour bioassays is lack of repeatability from laboratory to laboratory. Variations in temperature within and among growth chambers or incubators are often blamed for the lack of consistency. A major advantage of the ethanol-based vigour assays is that they are relatively insensitive to variations in temperature. Tests at 19, 23 and 27 °C showed that high- and low-vigour seed can be resolved over this temperature range (Figure 18). At 19 °C, though, 31 h was required for a full blue colour to develop for low-vigour seed compared to 24 h at 23 and 27 °C (compare Figures 18 and 19). The colour developed at 23 and 27 °C remained stable up to 48 h (compare Figures 18 and 20). The assays shown in Figures 18 - 20 were performed with carefully selected high- and low-vigour seed lots. The high-vigour lot was from a variety that produced relatively high ethanol emissions compared to other high-vigour seed, whereas the low-vigour lot was one that produced relatively low ethanol emissions compared to other low-vigour seed. This selection of seed minimized the difference in ethanol emissions between high- and low-vigour, and, therefore, challenged the capability of the assay to resolve the seed types. It is anticipated that, in most cases, 24 h will be adequate incubation time for the assay. Only if the colour is intermediate between yellow and blue at 24 h will there be a need to wait longer to determine if a full blue colour will develop. If a full blue colour does not develop by 48 h, then the sample is high vigour.

The results in Figures 18-20 also show that variation in starting seed moisture has little effect on the assay results. Little effect can be observed in the results shown. However, there is a trend for high moisture seed (10.4 %) to develop colour slightly faster and slightly more intensely than low moisture seed (4.4 %).

Accuracy/Reliability of the On-farm Assay

The reliability of the on-farm assay is being determined by assaying sample sets 2 and 3 and comparing the results with those obtained by gas chromatography and by hydroponics bioassay. This work is currently underway with existing resources. In addition, we plan to evaluate a smaller number of samples in field plots over three years and correlate field performance with the hydroponics, gas chromatography, on-farm and instrumental assays. The field work will require additional funding for which an application has been submitted to the Canola Council of Canada.

INSTRUMENTAL CANOLA SEED VIGOUR ASSAY

Although a simple colour test will be convenient for producers who may only want to test two or three samples, seed processors, seed merchants, seed laboratories and others associated with the canola industry may find a simple instrumental assay more efficient. In addition to efficiency, an instrumental assay will be able to provide quantitative data that may be more accurate and precise than colour change information. It will be advantageous for the seed industry to be able to test seed with a method based on the same principle as the on-farm test, so that the vigour of a batch of seed can be determined before it is released onto the market. Ultimately, of course, the rejection of poor vigour seed lots is the objective of seed vigour testing. It is expected that the combination of the on-farm assay and instrumental assays will achieve this goal.

After reviewing hand-held gas monitoring instruments, one was selected for study. This was the Pac III single gas monitor fitted with an XS EC Organic Vapors A sensor, part no. 6809115, from Draeger Canada Ltd., Mississsauga, ON (Figure 21). The monitor is not as specific nor as sensitive as a gas chromatograph; however, it appears to be suitable for a canola vigour assay. Figure 22 shows the determination of gaseous ethanol at concentrations suitable for the analysis of head space gas of moist seed using the Pac III. In order to use the Pac III for a seed vigour assay, it was necessary to modify the instrument by the addition of a flanged tube over its sensor. This tube allows direct measurement of ethanol gas in the assay bottles. It is only necessary to push the bottle onto the flanged tube to break a Teflon membrane and seal the bottle to the sensor. Figure 23 shows the Pac III in use during an experiment on instrumental assay conditions. At this time the assay procedure with the Pac III is still evolving. We expect it to be similar to the on-farm assay except that ethanol in the head space will be measured with the Pac III rather than a colourimetric reaction. We are currently investigating the possibility that the instrumental assay may be run within a normal working day. Once the technique has stabilised, the reliability of the instrumental vigour assay will be evaluated with sample sets 2 and 3.

TECHNOLOGY TRANSFER

Results of research were presented 1) at the Canola Council of Canada Seed Vigour Steering Committee meetings in March 2000, April, 2001, and March 2002; 2) at two BRC field days in July, 2001 and a BRC field research tour in July 2002; and 3) in a news release. The following media interviews were conducted on the canola seed vigour topic, especially the on-farm assay:

Manitoba Co-operator; Interviewer: Gordon Gilmore; Date: 01/06/14

Grain News; Interviewer: Larry Gompf; Date: 01/06/22
Western Producer**;** Interviewer Michael Raine; Date: 01/07/04
Brandon Sun; Interviewer: Lyndenn Behm; Date: 01/08/14
Canola Digest; Interviewer: Kelly Funke; Date: 01/08/27
Canola Guide; Interviewer: Jay Whetter; Date: 01/09/13
Canola in Canada; Interviewer: Janet Kanters; Date: 01/09/21
CBC radio (live, regional); Interviewer: unknown (contact: David White); Date: 01/09/28
Grain News; Interviewer: Larry Gompf; Date: 02/05/01

A provisional patent application was filed with the Canadian Patent Office on April 18, 2001 entitled "Assay for Seed Vigour". The final application (PCT) was filed with the Canadian Patent Office on April 18, 2002.

We are currently searching for an industry partner interested in manufacturing and marketing the on-farm assay kit and in promoting and marketing the instrumental assay.

LITERATURE CITED

Ahmed, SU and Zuberi, MI. Effect of seed size on yield and some of its components in rapeseed, *Brassica campestris* L. var. Toria. Crop Sci. 1973; 13:119-120.

Blackshaw RE. Soil temperature and moisture effects on downy brome vs. winter canola, wheat, and rye emergence. Crop Sci. 1991; 31(4):1034-1040.

Gorecki RJ; Harman GE, and Mattick LR. The volatile exudates from germinating pea (*Pisum sativum* cultivar Kriter) seeds of different viability and vigor. Can. J. Bot. 1985; 63: 1035-1039.

Gorecki RJ, Michalczyk DJ, and Esashi Y. Comparative studies on the anaerobic respiration in differently aged pea and cocklebur seeds. Acta Physiol. Plant. 1992; 14: 19-27.

Harman, GE, Nedrow, BL, Clark, BE and Mattick, LR. Association of volatile aldehyde production during germination with poor soybean and pea seed quality. Crop Sci. 1982; 22:712-716.

Johnson-Flanagan AM. Enhancement of low temperature germination in brassica. Alberta Agriculture Research Institute, Project no. AARI90M231; 1993 Jan.

Kondra ZP. Effects of planted seed size and seeding rate on rapeseed. Can. J. Plant Sci. 1977; 57:277-280.

Major DJ. Influence of seed size on yield and yield components of rape. Agron. J. 1977; 69:541-543.

May MJ, Vernoux T, Leaver C, Van Montagu M., and Inze, D. Glutathione homeostasis in plants: implications for environmental sensing and plant development. J. Exp. Bot. 1998; 49: 649-667.

Noctor G, Arisi A-CM, Jouanin L, Kunert KJ, Rennenberg H and Foyer CH. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J. Exp. Bot. 1998; 49: 623-647.

Pesis E and Ng TJ. The effect of seedcoat removal on germination and respiration of muskmelon (*Cucumis melo*) seeds. Seed Sci. Technol. 1986; 14: 117-126.

SAS Institute Inc. SAS Procedures Guide, Version 6, 3rd ed., SAS Institute Inc., Cary NC,

1990. Chapter 15.
Sword JT, Arthur LP and Hoekstra WG. Endotoxin and lipid peroxidation in vivo in selenium- and vitamin E-deficient and-adequate rats. J. Nutr. 1991; 121: 251-257.
Trawatha SE, TeKrony DM and Hildebrand DF. Relationship of soybean seed quality to fatty acid and C6-aldehyde levels during storage. Crop-Sci. 1995; 35(5): 1415-1422.
Wilson DO Jr and McDonald MB Jr. The lipid peroxidation model of seed aging. Seed Sci. Technol. 1986a, 14: 269-300.
Wilson DO Jr and McDonald MB Jr. A convenient volatile aldehyde assay for measuring soybean seed vigour. Seed Sci. Technol. 1986b, 14: 259-268.
Woodstock LW and Taylorson RB. Ethanol and acetaldehyde in imbibing soybean seeds in relation to deterioration. Plant Physiol. 1981; 67: 424-428.
Zar JH. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, NJ, 1984. Chapters 18 and 19.

Table 1. Elemental concentrations of 26 high- and low-vigour canola seed lots paired by variety (sample set 1).

Element	Units	High vigour (n = 13)		Low vigour (n = 13)	
		mean	SE	mean	SE
P	g kg^{-1}	6.25	0.32	6.34	0.21
K	g kg^{-1}	7.75	0.34	8.12	0.35
S*	g kg^{-1}	4.07	0.19	3.68	0.14
Ca	g kg^{-1}	3.08	0.14	3.32	0.07
Mg	g kg^{-1}	3.29	0.09	3.19	0.07
Zn	mg kg^{-1}	39.3	1.90	36.5	1.87
Cu	mg kg^{-1}	3.41	0.19	3.28	0.12
Fe	mg kg^{-1}	59.6	4.31	55.8	3.10
Mn	mg kg^{-1}	32.4	1.19	33.1	1.98
N*	g kg^{-1}	40.5	1.2	36.6	0.9

*High and low vigour seed is different ($P < 0.05$) with respect to element concentration.

Table 2. Volatile gas evolution by untreated canola seed (sample set 1) incubated for 24 h and 20 % moisture at room temperature. Seed lot pairs were divided into "high" (left) and "low" (right) vigour according to preliminary vigour estimations. The two seed lots within each pair were the same variety. All seed samples were then evaluated for vigour by a hydroponics bioassay based on growth of shoots and roots, rate of germination and rate of hypocotyl elongation during five days from start of imbibition. The "high"-vigour lot in each pair was assigned a bioassay vigour rating of 100 and the "low"-vigour lot was assigned a vigour rating as a percentage of its paired "high"-vigour lot. Results are means of 3 determinations each for bioassay and gas analysis.

Seed lot Pair	High-vigour lots				Low-vigour lots			
	Bioassay %	Head space gasses			Bioassay %	Head space gasses		
		EtOH	AA	E		EtOH	AA	E
10	100	20,389	5,399	0	103	34,148	9,653	0
12	100	5,441	1,872	0	89	16,691	3,390	0
8	100	28,940	6,806	0	79	274,664	50,894	0
6	100	28,391	8,143	0	79	24,240	4,870	0
7	100	70,172	10,012	0	77	6,267,588	243,643	22,647
11	100	27,780	8,703	0	74	3,097,329	233,091	56,990
9	100	22,667	4,427	0	74	1,125,623	138,339	7,573
13	100	11,757	2,145	0	73	129,176	19,846	0
2	100	300,722	0	4,591	71	6,184,369	89,264	50,602
5	100	38,710	9,463	0	67	4,208,946	262,515	32,092
3	100	75,696	16,472	0	65	6,426,057	333,208	25,685
AC Excel	100	5,724	2,420	0	57	1,439,619	73,715	19,648
4	100	16,354	3,113	0	55	5,030,866	407,121	78,330

*Definitions: EtOH = ethanol; AA = acetaldehyde; E = Unknown E.

Table 3. Correlations between hydroponics bioassay results (seedling fresh weight) and quantities of low-molecular weight volatiles found in head space gas of canola seed (sample sets 2 and 3) after 24 h of incubation at room temperature and 20 % moisture.

Head space gasses	n*	Correlation with seedling fresh weights	
		Correlation coefficient (r)	P
Ethanol⊥	151	-0.81	< 0.0001
Unknown E§	151	-0.66	< 0.0001
Acetaldehyde⊥	150	-0.70	< 0.0001
Unknown B§	151	-0.36	< 0.0001
Dimethyl sulphide⊥	150	0.24	0.003
Pentane§	147	0.09	0.27
Unknown C⊥	149	-0.04	0.65
Unknown F⊥	151	0.001	0.99

*Numbers less than 151 indicate that one or more outliers were omitted.
⊥Data transformed by square root.
§Data transformed by natural log.

Table 4. Comparisons of Pearson correlation coefficients (r) between several seed categories within sample sets 2 and 3.*

Category name	r	n	Category name	r	n	Difference between r
Untreated	-0.85	75	treated	-0.73	76	NS
			F	-0.76	44	NS
			F + I	-0.71	22	NS
Non-hybrid	-0.80	128	hybrid	-0.84	23	NS
Conventional genetics	-0.86	37	GM -- all	-0.78	54	NS
			GM -- LL	-0.85	12	NS
			GM -- RR	-0.70	32	NS
			MD -- SM	-0.77	19	NS

*F = fungicide; F + I = fungicide plus insecticide; GM = genetically modified; LL = Liberty Link; RR = Roundup Ready; MD = mutagenically derived; SM = Smart System; NS = not significant ($P > 0.05$).

Table 5. Correlations between various vigour tests and hydroponics bioassay results as well as ethanol emissions for a set of 17 canola seed samples.

Vigour test	Pearson correlation coefficients*	
	s assay	Ethanol emission
Field fresh weight 14 d after planting	0.79	-0.78
Field fresh weight 21 d after planting	0.80	-0.77
Field fresh weight 28 d after planting	0.83	-0.71
Pre-chill test⊥	0.77	-0.77
Special cold test§	0.84	-0.76
Germination after 4 d on moist blotting paper	0.89	-0.81
Germination after 5 d on moist blotting paper	0.74	-0.79
Germination after 6 d on moist blotting paper	0.68	-0.72
Germination after 7 d on moist blotting paper (standard germination test)	0.64	-0.68

*All coefficients in this table are statistically significant ($P < 0.05$).

⊥Seed was planted in a potting soil, chilled at 5 °C for 7 d, than warmed to 25 °C during 16-h light periods and 15 °C during 8-h dark periods for 5 d, after which the number of emerged seedlings was counted.

§Seed was planted in potting soil and sand mix (1:1), and maintained at 8 °C during 8-h light periods and 16-h dark periods for 14 d, after which the number of emerged seedlings was counted.