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# Decomposition of canola stubble by solid-state fermentation with *Cyathus olla.*

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*Cyathus olla* is being studied for its potential as an inoculant to accelerate decomposition of canola stubble, and hence to reduce the incidence of stubble-borne diseases of this crop. Stubble infested by *C. olla* appears soft and macerated, but the extent of decomposition incited by this fungus is not known. Composition of stubble fiber of five canola cultivars was determined with the Goering Van Soest method of fiber determination. Sterile canola (cv. Cyclone) substrate was inoculated with *C. olla* and incubated at 25°C for 45 days, followed by fiber analyses to detect changes in the fiber content. All cultivars were variable with respect to cell wall composition. Canola substrate inoculated with *C. olla* f. *brodiensis* had 60.6% of the original lignin remaining after incubation, compared to 65.9% for the substrate incubated with *C. olla* f. *olla* and 71.8% with *C. olla* f. *anglicus*. Hemicellulose content was reduced as only 75.3, 78.6, and 81.6% of the original hemicellulose content remained after incubation with *C. olla* f. *brodiensis*, *C. olla* f. *olla*, and *C. olla* f. *anglicus*, respectively. Cellulose was also degraded, and the neutral detergent soluble fraction increased. *Cyathus olla* was capable of degrading lignin *in vitro,* but field testing must follow to assess its decomposing activity under natural conditions.

**[Décomposition du chaume de canola par fermentation en milieu solide avec le *Cyathus olla*]**

Le *Cyathus olla* est étudié pour sa valeur comme inoculant pour accélérer la décomposition du chaume de canola, et ainsi réduire, dans cette culture, la fréquence des maladies véhiculées par le chaume. Le chaume envahi par le *C. olla* devient mou et macéré, mais l'importance de la décomposition induite par ce champignon est inconnue. La composition de la fibre du chaume de cinq cultivars de canola a été déterminée par la méthode de caractérisation des fibres de Goering Van Soest. Du substrat stérile de canola (cv. Cyclone) a été inoculé avec le *C. olla* et incubé à 25°C durant 45 jours avant que les fibres ne soient analysées pour détecter des modifications dans leur composition. Tous les cultivars variaient quant à la composition de la paroi cellulaire. Après incubation, le substrat de canola inoculé avec le *C. olla* f. *brodiensis* avait conservé 60,6 % de la

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lignine de départ, par rapport à 65,9 % pour le substrat incubé avec le *C. olla* f. *olla* et 71,8 % avec le *C. olla* f. *anglicus*. Le contenu en hémicellulose a été réduit puisque seulement 75,3, 78,6, et 81,6 % du contenu d'origine en hémicellulose a persisté après incubation avec respectivement le *C. olla* f. *brodiensis*, le *C. olla* f. *olla*, et le *C. olla* f. *anglicus*. La cellulose a aussi été dégradée, et la fraction soluble avec un détergent neutre a augmenté. Le *Cyathus olla* a été capable de dégrader la lignine *in vitro*, mais des essais au champ doivent maintenant être entrepris pour évaluer son activité de décomposition en conditions naturelles.

## INTRODUCTION

*Cyathus olla* (Batch) ex. Pers. (Nidulariaceae) is a white-wood rotting bird's nest fungus, commonly found colonizing and fruiting on the stubble of canola (*Brassica napus* L., *B. rapa* L.) in northern and central Alberta, Canada. This fungus is being studied for its potential as a microbial inoculant to accelerate decomposition of canola stubble in the field and to reduce the incidence of stubble-borne diseases like blackleg caused by *Leptosphaeria maculans* (Desm.) Ces. de N., and blackspot caused by *Alternaria brassicae* (Berk.) Sacc. The basal stem and root of canola are woody and resistant to decomposition, and may provide an overwintering site and food source for pathogens for many years.

Recently, the lignin-degrading enzyme system of *C. olla* was studied (Shinners-Carnelley, Szpacenko, Tewari, and Palcic, unpublished). Enzymes were isolated and identified from canola stubble after solid-state fermentation with *C. olla.* These enzymes included manganese peroxidase, laccase, and aryl-alcohol oxidase (Shinners-Carnelley, Szpacenko, Tewari, and Palcic, unpublished). *Cyathus olla* has been shown to secrete oxalic acid and form calcium oxalate crystals when the fungus colonizes canola residue (Tewari *et al.* 1997). Oxalic acid sequesters calcium from the cell walls of the substrate and chelates with it to form calcium oxalate crystals. The removal of calcium from cell walls renders the substrate susceptible to enzymatic degradation (Bateman and Beer 1965). Oxalic acid has also been implicated in the regulation of lignin-degrading enzyme system of white-rot fungi (Shimada *et al.* 1994). Presence of a lignin-degrading enzyme system and secretion of oxalic acid indicates that *C. olla* plays an active role in decomposition of canola stubble.

In addition to this potential application of this fungus, there are many reports to assess the use of white-rot fungi to delignify plant residues and to improve digestibility of this lignocellulosic material (Akin *et al.* 1995; Chen *et al.* 1995; Karunanandaa *et al.* 1995; Wicklow *et al.* 1984). More recently, delignification of rape residue has been studied in relation to the potential use of rapeseed as a food crop in controlled ecological life support systems (CELSS) (Kohlmann *et al.* 1995). Biological treatment of rape residue may convert lignocellulose and increase the edible portion of the residue.

For these potential applications, the ability of *C. olla* to degrade canola stubble should be confirmed and quantified. The basal stem and root of canola are woody, and slower to decompose compared to stem portions (Blenis *et al.* 1999). However, the proportion of structural cell wall components like lignin, cellulose, and hemicellulose have not been determined for canola root. In addition, much morphological and molecular variation has been observed in *C. olla* (Shinners and Tewari 1998). It is not known if the three forms of this fungus commonly found on canola stubble vary with respect to lignin degradation.

The objectives of this study were to determine the structural composition of the basal stem and root of canola, and to quantify changes in cell wall components of this canola substrate

after solid-state fermentation with three forms of *C. olla.*

## MATERIALS AND METHODS

### Plant material

Five cultivars of *Brassica napus* including Westar, Alto, Cyclone, Legacy, and Q2 were selected for this experiment. Field plots were planted in a randomized block design at the Edmonton Research Station, University of Alberta, Edmonton, Alberta in May 1998. After harvest in September 1998, standing stubble was dug from the plots to collect the root and stem portions. The stubble was dried at 60°C for 48 h and stubble pieces were cut and separated into root and basal stem, and main stem pieces. Root and basal stem portions were ground in a Wiley mill.

### Compositional analyses

Ground root and basal stem material was further ground to a fine powder (approximately 1 mm particle size) in a coffee grinder prior to analyses. The Goering Van Soest (Goering and Van Soest 1970) method of fiber analysis was performed with the filter bag technique and the Ankom$^{200}$ Fiber Analyzer (ANKOM Company, Fairport, New York). Fractions determined included: i, neutral detergent fiber (NDF); ii, acid detergent fiber (ADF); iii, cellulose; iv, lignin; and v, hemicellulose. Hemicellulose was calculated as the difference between NDF and ADF, and lignin was the fraction that remained after digestion in 72% $H_2SO_4$ (Klason lignin), minus the ash component that remained after 10 h at 500°C. Total nitrogen (N) was determined by the LECO nitrogen analysis system (LECO Instruments Limited, Mississauga, Ontario).

### *Cyathus olla* accessions and solid-state fermentation

Fifteen accessions of *C. olla,* five of each of *C. olla* f. *olla, C. olla* f. *anglicus,* and *C. olla* f. *brodiensis* (Shinners and Tewari 1998), were used in this study. All accessions were collected from northern and central Alberta, Canada (Table 1), and maintained on potato dextrose agar (PDA). Accessions were selected based on the ability to degrade a model lignin substrate (Shinners-Carnelley and Tewari, unpublished).

Fermentations were carried out in 250 mL Erlenmeyer flasks, each containing 3 g of ground canola substrate (cv. Cyclone), and moistened with 15 mL of $dH_2O$. The flasks were closed with a cotton plug, and covered with alumi-

**Table 1. *Cyathus olla* accessions used in this study**

| Form | Accession ID | Collection location |
|---|---|---|
| *C. olla* f. *olla* | PR 5 | Canada, Alberta, NW 9-78-21-5 [a] |
| *C. olla* f. *olla* | PR 6 | Canada, Alberta, SW 35-79-22-5 [a] |
| *C. olla* f. *olla* | PR 10 | Canada, Alberta, SE 5-78-21-5 [a] |
| *C. olla* f. *olla* | PR 15 | Canada, Alberta, SW 2-79-22-5 [a] |
| *C. olla* f. *olla* | Beau | Canada, Alberta, Beaumont |
| *C. olla* f. *anglicus* | Ravine | Canada, Alberta, Edmonton |
| *C. olla* f. *anglicus* | PR 1 | Canada, Alberta, SE 12-79-21-5 [a] |
| *C. olla* f. *anglicus* | PR 18 | Canada, Alberta, NW 16-77-21-5 [a] |
| *C. olla* f. *anglicus* | PR 22 | Canada, Alberta, SE 6-78-20-5 [a] |
| *C. olla* f. *anglicus* | PR 25 | Canada, Alberta, NE 33-77-20-5 [a] |
| *C. olla* f. *brodiensis* | PR 3 | Canada, Alberta, SW 24-78-20-5 [a] |
| *C. olla* f. *brodiensis* | PR 7 | Canada, Alberta, SW 6-80-20-5 [a] |
| *C. olla* f. *brodiensis* | PR 19 | Canada, Alberta, NW 10-77-21-5 [a] |
| *C. olla* f. *brodiensis* | PR 23 | Canada, Alberta, Falher |
| *C. olla* f. *brodiensis* | PR 27 | Canada, Alberta, SE 26-78-21-5 [a] |

[a] Legal descriptions within the Municipal district of Smoky River, Alberta, Canada.

num foil to prevent moisture loss, then autoclaved for 30 min at 121°C. Sterilization was repeated twice, at 24 h intervals to ensure complete sterilization and to eliminate the possibility of fermentation by stubble colonists other than *C. olla.*

The flasks were inoculated with six 2 mm x 2 mm agar plugs cut from the edge of 1-wk-old cultures on PDA of the accessions of *C. olla.* Multiple plugs were used to decrease the time required to achieve thorough colonization of the substrate. Because of the small size of the plugs, the contribution of this material to the final analysis was considered insignificant. Three flasks were inoculated with each accession. Three non-inoculated flasks served as the control treatment. All flasks were incubated at 25°C in the dark for 45 d. To prevent the substrate from drying out, 1 mL of sterile water was added to each flask at 10, 20, and 30 d following inoculation. After 45 d, the flasks, including contents, were dried at 100°C for 48 h, and loss in dry matter was determined by calculating the difference in the final dry weight compared to the initial weight prior to fermentation. The contents of each flask were ground in a coffee grinder, and then the compositional and N analyses were performed, as previously described. The average of three sub-samples was calculated for each flask. This entire growth experiment was repeated once and both experiments were analyzed together.

### Statistical analyses

The cultivar compositional analysis was analyzed as a randomized block design using the General Linear Models (GLM) procedure of SAS 6.0, and the means were separated with Tukey's test at the 5% level of probability. For the fermentation study, one flask represented one experimental unit, and there were three flasks for each accession and the control. The data were analyzed as a nested design with accessions nested within the three forms of *C. olla.* The GLM procedure and Tukey's test were also used for the analysis of this experiment. All values were represented as a percent of the dry matter of each sample.

## RESULTS

### Structural composition of canola

In analyses of the five cultivars, each was different in cell-wall composition (Table 2). The NDF values ranged from 70.1-77.7% of the total dry matter. The cv. Cyclone had a significantly higher NDF content than the other four cultivars. This cultivar also had the highest percentage of cellulose and hemicellulose, but cv. Westar had the highest percentage of lignin. Surprisingly, cv. Q2 had the lowest NDF value (70.1%). This cultivar rates excellent with respect

**Table 2. Composition (% dry matter) of root and basal stem tissue of *Brassica napus* cultivars determined by fiber and N analyses**

| Component | Q2 | Cyclone | Legacy | Alto | Westar | Standard Error |
|---|---|---|---|---|---|---|
| NDF [a] | 70.1 b | 77.7 a | 74.1 ab | 70.3 b | 72.0 b | 0.51 |
| NDS [b] | 28.4 a | 21.2 b | 24.8 ab | 28.4 a | 27.1 a | 0.43 |
| ADF [c] | 52.8 c | 59.1 a | 56.4 ab | 53.6 bc | 55.6 abc | 0.14 |
| Cellulose | 39.4 b | 44.5 a | 43.3 a | 39.4 b | 40.1 b | 0.35 |
| Lignin | 13.4 c | 14.5 b | 13.1 c | 14.2 b | 15.5 a | 0.14 |
| Hemicellulose | 17.3 bc | 18.6 a | 17.7 ab | 16.7 c | 16.5 c | 0.18 |
| Ash | 1.3 a | 0.91 bc | 0.87 bc | 1.1 ab | 0.63 d | 0.05 |
| % N | 1.0 a | 0.90 b | 0.90 b | 1.0 a | 0.90 b | 0.02 |

[a] NDF, Neutral detergent fiber. [b] NDS, Neutral detergent solubles. [c] ADF, Acid detergent fiber.
Means with the same letter within rows are not significantly different ($P = 0.05$) according to Tukey's test.

to straw strength, and it was anticipated that it may have a higher proportion of NDF compared to other cv. such as Legacy (74.1% NDF) and Alto (70.3% NDF) which are rated as good and fair, respectively. Nitrogen content was significantly different in some of the cultivars used in this experiment. However, biologically these differences in N values did not appear to be important considering the small percentage of this fraction (0.9-1.0% N) and the experimental error involved in the methodology.

## Solid-state fermentation by *C. olla*

Mycelium covered the entire surface of the substrate by 10 d after inoculation. The non-inoculated control flasks remained sterile throughout the 45 d incubation period. After solid-state fermentation with *C. olla*, the dry matter content of all inoculated material was significantly reduced compared to the non-inoculated control (Table 3). *Cyathus olla* f. *brodiensis* produced the greatest reduction in dry matter followed by *C. olla* f. *olla* and *C. olla* f. *anglicus*. Loss in dry matter for all treatments was adjusted by 5.7% to account for the higher temperature at which the samples were dried following solid-state fermentation. This percentage was determined from the loss in dry matter of the non-inoculated controls. The lignin fraction of all the fermented substrate was reduced, and varied significantly between the forms of *C. olla*. Canola incubated with *C. olla* f. *brodiensis* had 10.9% lignin compared to 11.5 and 12.5 for *C. olla* f. *olla* and *C. olla* f. *anglicus*, and 15.5% for the control (Table 3). *Cyathus olla* f. *brodiensis* was the most effective at lignin degradation, as only 60.6% of the original lignin mass remained after fermentation (Table 4). *Cyathus olla* also metabolized hemicellulose, as this fraction was significantly reduced compared to the

**Table 3. Chemical composition (% of dry matter) of canola after 45-d solid-state fermentation with three forms of *Cyathus olla***

| Component | Control | *C. olla* f. *anglicus* | *C. olla* f. *olla* | *C. olla* f. *brodiensis* | Standard Error |
|---|---|---|---|---|---|
| Loss in dry matter | 0.0 a | 11.5 b | 11.9 b | 14.3 c | 0.20 |
| Lignin | 15.5 a | 12.5 b | 11.5 c | 10.9 d | 0.15 |
| Hemicellulose | 19.0 a | 17.4 b | 16.8 c | 16.6 c | 0.16 |
| Cellulose | 45.6 a | 47.5 b | 47.6 b | 47.9 b | 0.24 |
| NDS [a] | 18.7 a | 21.3 b | 22.5 c | 23.4 d | 0.29 |
| Ash | 0.85 a | 0.94 a | 1.3 b | 0.8 a | 0.07 |
| % N | 0.94 b | 0.95 b | 1.1 a | 0.91 b | 0.02 |

[a] NDS, Neutral detergent solubles.
Means with the same letter within rows are not significantly different ($P = 0.05$) according to Tukey's test.

**Table 4. Chemical composition of canola expressed as a percentage of original dry mass following 45-d solid-state fermentation with three forms of *Cyathus olla***

| Component | Control | *C. olla* f. *anglicus* | *C. olla* f. *olla* | *C. olla* f. *brodiensis* |
|---|---|---|---|---|
| Loss in dry matter | 0.0 | 11.5 | 11.9 | 14.3 |
| Lignin | 100 | 71.8 | 65.9 | 60.6 |
| Hemicellulose | 100 | 81.6 | 78.6 | 75.3 |
| Cellulose | 100 | 92.8 | 92.8 | 90.6 |
| NDS [a] | 100 | 101.5 | 106.9 | 107.9 |
| Ash | 100 | 98.5 | 135.9 | 81.1 |
| % N | 100 | 90.0 | 104.0 | 83.5 |

[a] NDS, Neutral detergent solubles

control. Cellulose did not appear to be degraded by *C. olla* when calculated as a percentage of the composition of the fermented sample. However, when expressed as a percentage of the original dry matter, cellulose slightly decreased. The neutral detergent soluble (NDS) fraction of all inoculated treatments increased significantly compared with the control. This fraction measures cell contents including soluble carbohydrate, starch, organic acids, protein, and pectin (Van Soest 1982), but does not differentiate between plant and fungal tissue. Fungus-mediated breakdown of structural cell wall components increases the soluble carbohydrate content of the NDS fraction, but as the fungi grow, these carbohydrates are incorporated into the fungal biomass.

The N content of the fermented samples did not differ greatly between treatments. Statistically, the canola inoculated with *C. olla* f. *olla* had a significantly higher N content when compared with the other forms of *C. olla* and the control (Table 3). However, for the reasons discussed previously with respect to N content in the cultivar experiment, this result does not appear to be biologically significant. In addition, the fermented samples also contained fungal biomass, and the N content represented in Table 3 does not distinguish between plant and fungal N.

## DISCUSSION

The cultivars examined in this study varied in structural composition and fiber content. The composition of the basal stem and root of canola have not been previously reported, but Kohlmann *et al.* (1995) determined that rape stems and siliques were composed of 38 and 35% cellulose, 10 and 12% hemicellulose, and 18 and 18% lignin on a dry weight basis, respectively. The root and the basal stem of the plant are more resistant to decomposition compared to stems and siliques, and it is assumed that the proportion of lignin and structural carbohydrates would be greater in this more resistant tissue. This assumption is supported by the data obtained for cellulose and hemicellulose but not for lignin content of root and basal stem tissue in the current study. However, these comparisons can only be generalized since they are based on the results of two independent experiments.

The lignin content of cv. Cyclone was 14.5% in the cultivar experiment, but when this canola tissue was used as the substrate and non-inoculated control for the solid-state fermentation experiment, the lignin content of cv. Cyclone was determined to be 15.5% of the dry matter. This is not a large discrepancy in data, but may have resulted from the formation of Maillard products. When exposed to heat greater than 60°C, carbohydrates may degrade, resulting in condensation of sugar residues with amino acids, polymerization, and subsequent formation of lignin-like compounds, referred to as Maillard products, via the Maillard reaction (Van Soest 1982). In the fermentation experiment, the substrate was subjected to heat sterilization to eliminate other stubble microorganisms that would have interfered with the colonization and decomposition of canola by *C. olla.* During this process, the Maillard reaction may have occurred and resulted in a slightly higher lignin content for the fermented samples.

Fermentation with every form of *C. olla* resulted in a decrease in the lignin fraction of the substrate. This result complements field observations of macerated stubble infested by *C. olla* (Tewari and Briggs 1995) and the recent finding of a lignin-degrading enzyme system in this species (Shinners-Carnelley, Szpacenko, Tewari, and Palcic, unpublished). Decayed samples had a significantly lower lignin content compared to the control. However, Horwath and Elliott (1996) caution that the Klason method of lignin determination, as used in this experiment, may be a conservative estimate of this fraction.

Fermented samples had a significantly lower hemicellulose fraction compared with the control. This was interpreted that *C. olla* metabolized this carbohydrate as an energy source during the incubation period. Cellulose

concentration in the fermented canola increased as a result of fermentation, but overall, the mass decreased slightly.

As degradation of crop stubble occurs in the field, N is metabolized from the residue and immobilized in the microbial biomass or mineralized and made available for plant growth. Ultimately, this results in a lower N content of the stubble. In this experiment, percent N in the fermented samples was not significantly different from the control, with the exception of *C. olla* f. *olla* which had a higher N content following fermentation. As previously mentioned, the methods used in this experiment did not allow for separation of plant and fungal biomass, and subsequently also plant and fungal N.

Bird's nest fungi are white-wood rotters ecologically specialized to degrade lignin. We found that *C. olla* was capable of degrading canola stubble *in vitro*, and also we demonstrated the variability within this species in stubble-degrading capabilities. *Cyathus olla* f. *brodiensis* was most effective to reduce the overall dry weight, and the lignin and hemicellulose fractions of canola. This is a significant finding since *C. olla* f. *brodiensis* has only recently been designated as a new form of *C. olla* (Shinners and Tewari 1998). This form is morphologically and molecularly distinct from the other previously described forms, and is also biochemically unique.

Future research on *C. olla* as a microbial stubble inoculant to accelerate canola stubble decomposition is warranted. The extent of *C. olla*-mediated decomposition in the field under natural conditions needs to be determined in the presence of other stubble colonists, and pathogen populations. The ability of *C. olla* to delignify canola residue may also be beneficial as a biological treatment to improve the nutritive value of this highly lignocellulosic material.

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