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BIOCHEMICAL STUDIES OF THE WHEAT (*TRITICUM AESTIVUM L)*

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ABSTRACT

Tricin (5,7,4'-trihyroxy-3',5'-dimethoxyflavone), a naturally occurring compound, is a characteristic constituent of the grass family, including cereal grain plants, and has been isolated from wheat. Apart from being a powerful antioxidant, antimutagenic, and anti-inflammatory agent, tricin has been considered as an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells and colon cancer cells. However, its high commercial price as a pure compound may hinder further experimentation. Wheat is considered one of the main staple foods in Canada and worldwide, and is the most widely adapted crop to abiotic stresses. The main aim of this study is to investigate the effects of abiotic stress factors, such as cold, drought, and salt treatments, among others, on the biosynthesis and accumulation of tricin in different parts of wheat *(Triticum aestivum L)*, with aim of defining an optimum source for tricin production in this important crop. This thesis consists of four research chapters. The first chapter focuses on an investigation of the phenolic profile of two varieties of wheat (*Triticum aestivum* L*)* leaves grown under normal and cold stress conditions. The leaf 'phenolomes' were established for two varieties: the winter wheat (*Triticum aestivum L.* var Claire) and spring wheat (*Triticum aestivum L.* var, Bounty) using a combination of HPLC-ESI-MS techniques. Phenolic compounds accumulated at a higher level in the Claire than in the Bounty variety, and detected in significant amounts in the apoplast compartment. The accumulation of a mixture of beneficial flavonoids in iv cold-acclimated wheat leaves attests to its potential use as an inexpensive supplement of a health-promoting component to the human diet. The second chapter describes the distribution of tricin in different parts of wheat with the aim to designate a rich source for its utilization. Winter wheat husk was identified as the most valuable part. Its tricin content is considered the highest in any plant materials suggesting the use of winter wheat husk as a good source of tricin. Moreover, the potential anticancer effect of tricin on two cancer cell lines was evaluated where it was revealed to have a selective anticancer effect. In the third chapter, the selective anticancer effect of several methylated phenolic and flavonoids compounds were tested *in vitro* on cell cultures, using a LDH-spectrophotometer method to assess the viability of the cell lines. Several candidates were found to possess a remarkable antitumor activity on these malignant cell lines, such as trimethyltricetin, a tricin derivative that exhibited a superior selective activity against human adenocarcinomic alveolar basal epithelial cells (A-549). In the last chapter, the biosynthesis of tricin is discussed. The expression and the enzyme activity of TaOMT2, the enzyme that catalyzes the methylation of tricetin to tricin, were measured at different wheat developmental stages and in response to different abiotic stresses such as cold, salt and drought. The significant accumulation of tricin in the inflorescences suggests that tricin may play a role in protecting the seeds against biotic and abiotic stresses.

KEYWORDS: Tricin, chemopreventive, *Triticum aestivum*, trimethyltricetin.

INTRODUCTION

Wheat is considered one of the most important crops in the world. According to the UN Food and Agriculture Organization (**FAO***)*, Canada is ranked sixth worldwide for the production of wheat and the third for its exportation, however, fluctuations in Canadian production due to weather can have a significant impact on world wheat supply.

Wheat is a good source of protein, minerals, B-group vitamins and dietary fiber, it provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally (Kumar, 2011). It is easy to store and transport and can be processed into various types of food. It is cultivated over a wide range of climatic conditions.

There are two varieties of wheat: spring wheat planted in the spring season and harvested in late summer or early fall and a winter wheat variety sown in the fall and harvested in the next early summer.

Wheat's Phenolic Compounds

Wheat is not only considered as the main staple food in many countries, but also a potential source of natural products with nutraceutical and/or pharmaceutical importance. Its high content of phenolic compounds confers significant antioxidant, anticarcinogenic and health-promoting properties (Craig, 1999). In fact, the antioxidant and radical scavenging activities of wheat bran extract were reported to be higher than those of the synthetic compound, butylated hydroxytoluene (BHT) (L. Brindzova et al., 2009).

Investigations of phenolic compounds in wheat are limited (Asenstorfer et al., 2006; Cavalière et al., 2005; Feng and McDonald, 1989; Feng; and McDonald, 1989), and a comprehensive profiling of wheat phenolic compounds, especially in relation to environmental stresses is lacking (Estiarte et al., 1999; Nadeau et al., 1987; Olenichenko et al., 2008; Zagoskina et al., 2005).

Stress Factors and Tricin

Only a few investigations were reported on the effect of some stress factors on the level of tricin in plants, Among these, one recent study reported the effects of the herbicide 'safener' on wheat seedlings. This study showed the increase in tricin and ferulic acid and the reduction of apigenin, luteolin and isorhamnetin levels concomitant with an increase in *O*-methyltransferase activity toward these flavonoid substrates (Cummins et al., 2006). The results suggested that safeners, besides altering the capacity of wheat to metabolise herbicides and other xenobiotics, could selectively shift the metabolism of endogenous phenolics.

Dietary Fibers

Dietary fibers constitute an important part of the plant material that is resistant to digestion by human enzymes. They are predominantly non-starch polysaccharides, lignins, and may include other associated substances (Dhingra et al., 2011). Dietary fibers are categorized according to their water solubility into two types: soluble, well fermented fibers (pectin, gums and mucilage) that are readily fermented in the colon into gases and active byproducts, and insoluble, less fermented fibers that are metabolically inert, absorbing water such as cellulose, hemicellulose, lignin, psylium husk and other roughage (Antia and Abraham, 1997). The insoluble dietary fibers class is also known as 'Novel fibers', its use is considered newborn and is responsible for most of the health benefits attributed to the use of whole grains.

The Impact of Plant-Based Foods on Human Health

Diets rich in phytonutrients, or plant-based foods, are mostly associated with reduced risks of major chronic diseases (Martin et al., 2011 and refs. there in). To be able to implement the role of these phytonutrients in human life, the collaboration between plant scientists, in plant biochemistry, plant genetics and metabolic engineering and researchers in nutrition and pathology of chronic diseases was recently highly recommended (Martin et al., 2011). Such collaboration and contribution will allow the designation of foods that reduce the risk of chronic disease and explain how these foods work to impact human health.

The development of dietary agents for cancer chemoprevention offers a safer and attractive alternative in comparison to the use of pharmaceuticals such as nonsteroidal anti-inflammatory drugs and tamoxifen as cancer chemopreventive agents. Recently, this approach was discussed in order to establish a protocol for development of chemopreventive phytochemicals and the selection of the optimal clinical dose required (Scott et al., 2009).

Techniques Used In This Work

LC-MS Techniques

Nowadays, LC-MS represents a well-established, rapid and powerful technique for the analysis of many natural products.

Mass spectrometry (MS) offers great selectivity and sensitivity as a detection technique, coupling it with high performance liquid chromatography (HPLC) enables effective analysis of complex matrices like plant extracts. This is mainly due to its ability to detect, within the same analysis, all metabolites encountered in the extract.

In addition, the use of tandem mass spectrometry (MS/MS) results in a full structural analysis of mixtures without using the tedious time-consuming isolation procedures.

Multiple Reaction Monitoring Technique (MRM)

(MRM) is a selective and sensitive LC-MS-MS tandem mass technique in which each ionized compound gives a distinct precursor-to-product ion transition indicative of a particular compound in an extract. Peaks containing co-eluting compounds are resolved by monitoring of specific precursor-to-product ion transitions (Chiwocha et al., 2003; Pang et al., 2009; Segarra et al., 2006).

Time-of-Flight (TOF)

(TOF) is a fast and precise ionization technique. A TOF instrument provides accurate mass measurement within a few parts-per-million (ppm) of a molecule's exact true mass (Cotter, 2004).

Viability Test

Two different methods were carried out during this work in order to determine the viability of different cell lines

Lactate dehydrogenase (LDH)

Lactate dehydrogenase **(LDH)** is an ubiquitous enzyme present in a wide variety of organisms, including plants and animals. It catalyzes the reversible reduction of pyruvate into lactate, with the ultimate interconversion of NADH into NAD+ (Fig 1-14) (Henderson, 1984). In isolated organ systems, *in vitro* release of LDH by the

cells into the culture fluid has been used to determine injury of the cells.

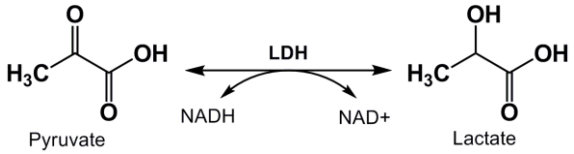


Figure 1: LDH reaction

The activity of lactate dehydrogenase is usually measured spectrophotometrically at 340 nm by one of the following two methods. Either the oxidation reaction of NADH with pyruvate and hence a decrease in the absorbance, or the reduction reaction of NAD+ with lactate resulting in an increase in the absorbance. Under standard conditions, one unit of enzyme catalyses the oxidation of NADH or reduction of NAD+ at 1 µmol per min (Markert, 1984).

In the current work the leakage of LDH was measured by monitoring the activity of lactate dehydrogenase with an aliquot of cell-free medium and compared to the total activity achieved after lysis of the cells (Moldéus et al., 1978).
Lysis of cells was done by treatment with Triton X-100 (0.5%). NADH (0.2 m*M* final concentration) and pyruvate (1.36 m*M* final concentration) were mixed with Krebs Henseleit buffer containing 2% albumin before being added to 25 µl of the cell-free medium once and to 25 µl of the cell-containing media after lysis. The rate of change in absorbance at 340 nm due to NADH oxidation was recorded.

FACS can (Fluorescence Activator Cells Sorter) Flow Cytometer
A flow cytometer is an instrument for detecting and measuring the amount of fluorescent dye on particles, and basically consists of one or more lasers for supplying excitation energy, and a series of filters and detectors for measuring the resultant fluorescent emissions. In this work a FACScan flow cytometer with CountBright™ absolute counting beads were used to measure viability of cells.

The Count Bright™ absolute counting beads are a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres.

(http://probes.invitrogen.com/media/pis/mp36950.pdf).
For absolute counts, a specific volume of the microsphere suspension is added to a specific volume of sample, so that the ratio of sample volume to microsphere volume is known. The volume of sample analyzed can be calculated from the number of microsphere events, and can be used with cell events to determine cell concentration. In general, at least 1,000 bead events should be acquired to assure a statistically significant determination of sample volume.

Cell concentration was calculated according to the following formula
A x C / B x D = concentration of sample as cells/µL;
Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50 µL)
D = volume of sample (µL)

Plant growth and acclimation conditions
Two wheat varieties (*Triticum aestivum*, 2nx6 = 42), a spring habit cultivar Bounty and a winter habit cultivar Claire, were grown in a controlled growth chamber as previously described (Danyluk et al., 2003). Plants were grown at 20 ºC under long days (LD), with 16-h photoperiod at a light intensity of 250 µmol m-2 s-1. For cold acclimation (CA), 7-49 day-old wheat plants were grown for 1, 10, 21 and 42 days at 4ºC under the same photoperiod and light conditions mentioned above.

Extraction of phenolic compounds
Fresh leaf samples (*ca.* 10 g) were ground in dry ice before extraction 3-times for 1hr with hot MeOH-H2O (85:15, v/v). The extract was concentrated in vacuo, to remove methanol, and the resulting aq. suspension was defatted with hexanes, followed by 3-times liquid-liquid extraction with EtOAc. The combined organic layers were concentrated, and the residue dissolved in a minimum amount of MeOH for analysis. For acid hydrolysis, the defatted aq. extract was hydrolyzed with 2M HCl (30 min; 95 ºC) in order to release the phenolic aglycones from their parent *O*-glycosides, followed by liquid-liquid extraction with EtOAc. The combined organic layers were concentrated under vacuo, and the resulting residue dissolved in MeOH for analysis.

HPLC and LC/ MS analyses
For quantification of total phenolic compounds, samples were applied to chromatographed on a Varian HPLC system equipped with a UV detector and a Varian XDB-C18 column (4.6×150 mm; particle size, 5µm) using a linear gradient of MeOH-1% HOAC in H2O (40:60, v/v) and a flow rate of 1 ml.min−1 for 30 min and a column temperature of 25ºC. The process was repeated at least three times, and three injections were analysed for each sample. This system was used only for the quantification step of total phenolic compounds, but not for quantification of individual compounds where the MRM technique was better used for this purpose, since it allowed the determination of minor components. Quantification for total phenolic compounds (Fig. 2-1) was carried out using the area under the curve method by calculating the summation of areas in relation to their UV absorbance, whereas quantification for individual compounds was performed using the MRM technique that allowed determination of minor compounds.

LC-MS analyses were carried using an Agilent 1200 HPLC system with binary pump, in-line degasser, high performance auto-sampler and thermo-stated column division, using a linear gradient of MeOH-0.1% HCOOH in H2O (40:60, v/v) for 30 min with a flow rate of 0.35 ml.min-1 on an Agilent SB-C18 column (2.1 ×30mm; particle size, 3.5µm), and a column temperature of 25ºC. The method was optimized with UV detection at 254 and 340 nm.

Two LC-ESI-MS systems were used: in System 1, the HPLC instrument was connected to an Agilent 6410 quadrupole (triple Q) mass spectrometer using electrospray ionisation in positive ESI mode with the following conditions capillary voltage: 3000 v, nebulizer pressure: 60 Psi, gas temperature 300ºC, drying gas: 5 L/min and a dwell time of 75 ms, and the data was processed using the Mass Hunter software. The same column was transferred to be used in system 2 where an identical HPLC instrument with the same conditions listed above was connected to another mass spectrometer consisting of an Agilent 6210 electrospray ionisation-time-of flight analyser (ESI-TOF) in positive ESI mode, at a capillary voltage of 4000V, nebulizer pressure of 35 Psi, gas temperature 350ºC, drying gas flow: 11.5 L/min and voltages of 125V and 60V for the fragmentor and the skimmer, respectively. The technical error and mass resolving power of the time-of-flight mass spectrometer in terms of mass accuracy was 2ppm, RMS, measured at the [M+H]+ ion of reserpine (m/z 609.2807) was used as an internal mass reference. When available, reference compounds were used to compare the corresponding retention times and mass spectral profiles of phenolic derivatives and flavonoids.

A quercetin calibration curve was constructed for quantification purposes, using different concentrations of 1.6µg.ml-1, 5µg.ml-1, 8.3µg.ml-1, 16.6µg.ml-1, 25µg.ml-1, and 166µg.ml-1 and 266µg.ml-1 quercetin.

For MS/MS and MRM analyses in system 1, the collision energy (CE) was optimized for each individual compound; available standards were injected several times with different energies of collision: 5eV, 10eV, 20eV, 25eV, 35eV and comparing their MRM peak area resulted from the MS/MS analysis for each case. The (CE) with the highest and more intense area was selected as the optimum one for the analysis, then tried again in both negative and positive modes of ionization. A (CE) of 35eV was applied as the optimum energy of collision for the analysis of flavonoids and coumarins, and 10eV for simple phenolic compounds and 25eV for conjugated amines, and the scan range was from 200 to 900 m/z. For each compound, MRM acquisition was carried out by monitoring transitions of the combination of the parent ion mass and the fragment ions of highest abundance.

Protein extraction and quantification

Protein was extracted in Tris-HCl, pH 7.0 containing 0.1% PVPP, followed by centrifugation at 10,000 rpm. It was quantified by the method of (Bradford, 1976) using bovine serum albumin as the standard protein.

***O*-Methyltransferase assays**

Protein was extracted from the plant material at 4°C. Wheat leaves were ground to a fine powder with dry ice and sand, before being homogenized with PBS buffer (pH 7.3). After centrifugation (14, 000xg) for 30 min, the supernatant was desalted on PD-10 and used directly for the assay of *O*-methyltransferase **(**OMT) activity against tricetin and 5-hydroxyferulic acid as external substrates. The enzyme assay consisted of 50µM S-adenosyl-L-methionine (AdoMet) containing 0.025 µCi of [3H] label, and protein extract (100 µg) and 200 µM tricetin or 5-hydroxyferulic acid (in 1% DMSO1) in a total volume of 100 µl. The reaction was initiated by addition of enzyme, incubated for 30 min at 30°C, and terminated by the addition of 6M HCl (10 µl). The methylated products were extracted with ethyl acetate, and a fraction was counted for radioactivity using a toluene-based scintillation fluid. Enzyme assays were carried out in triplicate, and the experiment was repeated at least twice. Control reactions were carried out without addition of protein for background correction.

1 DMSO: dimethylsulfoxide

Extraction of soluble apoplastic metabolites

Soluble apoplastic metabolites were extracted as described by (Vanacker et al., 1998). Freshly cut leaves (*ca*.5 g) of both non-acclimated and acclimated winter wheat were washed with distilled H2O, then immersed in Petri dishes containing 50 mM acetate buffer (50 ml) pH 4.5, 100 mM KCl and 2 mM CaCl2. The dishes were transferred to a vacuum dessicator and a vaccum of 270 kPa was applied in order to extract the apoplastic content. After centrifugation for 10 min at 2900*x*g and 4°C, the extract was lyophilized and the resulting powder used for both HPLC-MS/MS analysis and gel electrophoresis. The apoplastic purity was assesed by its protein pattern on SDS-PAGE and the absence of any trace of Rubisco that is usually used as indicator of cell leakage (Diaz-Vivancos et al., 2006).

RESULTS AND DISCUSSION

Identification of wheat phenolic compounds common to the winter and spring varieties

By means of two LC-ESI-MS protocols, a number of phenolic compounds were identified in the leaves of both varieties. The LC-TOF system was equipped with Agilent software that allowed calculating and generating the molecular formula of each compound according to its mass spectrum obtained during analysis, whereas the triple quadrupole MS/MS system was used to confirm the product ions. A direct comparison of the MS spectra obtained from both systems made it possible to confirm compound identification. Moreover, the high sensitivity of the MS-MS detector used allowed for identification of minor constituents with a high degree of fidelity. The fact that several phenolic compounds exhibited almost similar polarities and retention times made it difficult to

assign their corresponding peaks. However, the use of MS in combination with UV detection at 340 and 254 nm, in addition to the comparison of their spectral data with available reference compounds, allowed their identification with high degree of certainty. In addition, tandem mass spectrometry (MS-MS), exact mass measurements and fragmentation patterns were used together with literature data for the identification of compounds where reference samples were not available.

The application of several collision energies in the positive and negative modes to the sample was necessary to optimize a method with a consistent fragmentation pattern. The positive mode was compatible with almost all compounds. For optimum energy of collision, 10V and 25V were applied for simple phenolic derivatives and hydroxycinnamic acid conjugates, respectively, whereas 35V was chosen for other flavonoids and coumarin derivatives. This was performed through the application of tandem mass technique MS-MS in multiple reaction monitoring (MRM) mode and of exact mass measurement, both of these methods allowed the identification and quantification of most phenolic compounds.

MRM is a selective and sensitive LC-MS/MS technique in which each ionized compound gives a distinct precursor-to-product ion transition. Furthermore, peaks containing co-eluting compounds were resolved by monitoring for specific precursor-to-product ion transitions (Chiwocha et al., 2003; Pang et al., 2009; Segarra et al., 2006). However, closely related isomers could not be differentiated by this technique. Another analysis using the same conditions for HPLC was conducted on another instrument (ESI-TOF) in order to confirm the exact masses and empirical formulae of each of the identified structures.

Using these protocols allowed the separation and identification of 40 phenolic compounds in the extracts of both winter and spring wheat varieties. These consisted of two coumarin derivatives, eight simple phenolic compounds, ten hydroxycinnamoyl amides (HCAs) and 20 flavonoid derivatives (Table 2-1, Fig. 2-2). These results showed no qualitative differences observed in the phenolic profiles of both varieties under non-acclimated (control) conditions, (Figs. 2-1A and 2-1B).

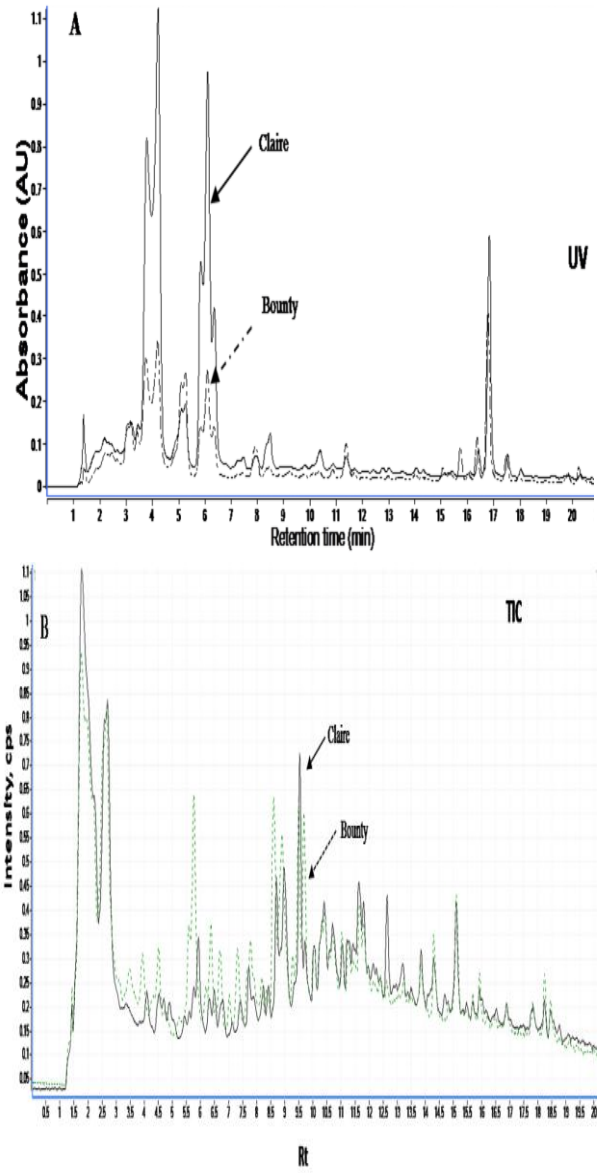


Figure 2: A, HPLC-UV; B, Total ion counts (TIC)-overlaid trace chromatograms of non-hydrolysable methanolic extract of leaves from 2-week-old Claire and Bounty.

The UV and TIC chromatograms were obtained using an Agilent SB-C18 column (2.1 ×30mm; particle size, 3.5µm), and a column temperature of 25℃; 40% to 90% MeOH in 0.1% HCOOH of linear gradient elution; flow rate 0.35 ml/min for 30 min.; injection volume, 10 µl; wavelength monitoring, 340 nm.

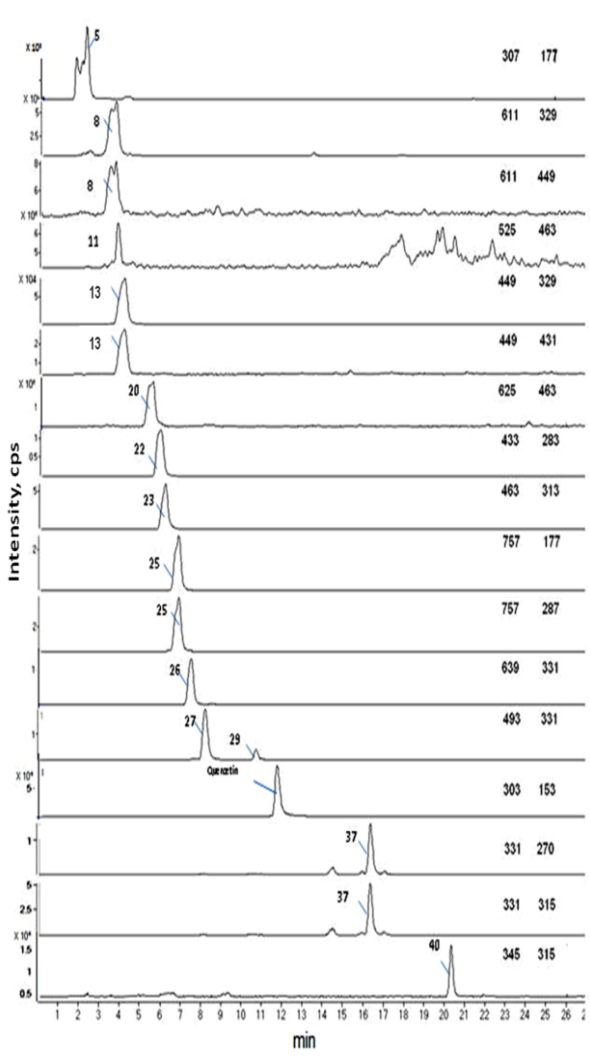


Figure 3: HPLC-UV-MS of methanol extract of leaves from 2-week-old winter wheat Claire.

*Reconstructed MRM chromatogram [MS-MS] of most abundant protonated molecules (parent ion [M+H]+ and its major ion peak fragments) in the positive mode resulting from LC-MS-MS analysis, showing MRM at: 307 -> 177 for compound (**5**), 611 -> 329, 611-> 449 for compound (**8**), 525 -> 463 for compound (**11**), 449 -> 329 for compound (**13**), 625 -> 463 for compound (**20**), 433 -> 283 for compound (**22**), 463 -> 313 for compound (**23**), 639 -> 331 for compound (**26**), 771 -> 463, 771 -> 625for compounds (**28**) and (**29**), 303 -> 153 for quercetin, 331 -> 315 for compound (**37**) and 345 -> 255 for compound (**40**)*

Hydroxycinnamoyl amides (HCAs)

The major HCAs identified in this study (Table 2-1) are *p*-coumaroylagmatine (**1**), caffeoylputrescine (paucine) (**2**), *p*-coumaroylputrescine (**3**), feruloylputrescine (**4**), *trans*-feruloylagmatine (**5**), *p*-coumaroylspermidine (**6**), *p*-coumaroyl-2-hydroxyputrescine (**7**), *bis*-dihydrocaffeoylspermine (**30**), dicaffeoylputrescine (**31**) and di-*p*-coumaroylputrescine (**34**).

Feruloylagmatine **(5),** was analysed using 25V collision energy, and its MRM transitions from 307 [M+H]+ to 177 at Retention time of 2.01 min. This data was identical to those obtained for a reference sample of feruloylagmatine **(5)**. The identification of small amounts of dihydrocaffeoylspermine **(30)** in the spring variety (Table 1) is surprising, since dihydro derivatives of phenylpropanoid compounds are reported to be of rare occurrence in plants (Anterola and Lewis, 2002; Lewis, 1999). However, the occurrence of several dihydrocaffeoyl polyamines in potato tubers was reported during metabolite profiling of the plant (Parr et al., 2005), which provides an exemplary evidence for the efficient use of LC-EIS-MS-MS protocols in investigating wheat metabolites.

Flavonoids

Among the 20 flavonoids identified in this work, six represented the major constituents. These include (% of total and µM quercetin equivalent.g-1): orientin (**12**) and iso-orientin (**13**) (42%, 0.47); vitexin (**21**) and iso-vitexin (**22**) (18.2%, 0.26); chrysoeriol 6-*C*-glucoside (**23**) (8.7%, 0.2) and tricin (**37**) (9 %, 0.21). Acid hydrolysis of methanolic extracts, that removed *O*-glycosidic linkages followed by LC-MS of the hydrolysis products, confirmed the natural occurrence of these *C*-glycosides in wheat leaves and allowed the quantification of tricin (**37)** as a free aglycone. These results are consistent with the most recently published LC-MS analysis for wheat leaves (Cavalière et al., 2005), among others, which reported the occurrence of these flavonoids as *C*-glycosides, except tricin 37 which occurred as an *O*-glycoside.

In cereals, such as wheat, maize, barley and rice, both 6-*C*- and 8-*C*-glycosides of luteolin and /or apigenin are most abundant (Brazier-Hicks et al., 2009; Cummins et al., 2006). These compounds were suggested to act as antibiotics, antioxidants, feeding attractants or deterrents (Brazier-Hicks et al., 2009; Gould and Lister, 2005), and as phytoalexins that were produced naturally in response to various stress conditions (Du et al., 2009; McNally et al., 2003). Furthermore, iso-orientin (**13**), the major phenolic compound in wheat was reported to act as anti-nociceptive and anti-inflammatory agent in rats and mice at doses of 15 and 30 mg.kg-1, without causing any apparent acute toxicity or gastric damage (Kupeli et al., 2004). In addition, there is ample evidence to suggest that luteolin **(36)** and its glycosides **(12)-(13)** might be used as cancer chemopreventive agents, or in chemotherapy (Lopez-Lazaro, 2009)& refs therein).

The relatively high abundance of iso-orientin (**13)** in wheat leaves, attests to its use as a potential source of active natural health-promoting compounds. Wheat leaves are considered as an edible part of the plant, and are used as a juice (wheatgrass juice) or added to several food products in North America and other parts of the world. These products are approved by the health authorities and sold by several established companies: 59

(http://www.greenhealthcanada.com/Benefits_of_Wheat grass.html; http://www.wheatgrass.ca/; http://www.ble-de-vie.com/english.html), among others.

Tricin (**37)** (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) and its *O*-glycosides **26, 27**, **29** were found to constitute about 9% of the total phenolic fraction in wheat leaves after acid hydrolysis. It is known to occur mainly in the grass family, including cereal grain plants, and has been isolated from rice, oat, maize and wheat (Wollenweber, 2008). Apart from being considered as a powerful antioxidant, antimutagenic and anti-inflammatory agent (Zhou et al., 2006) and refs. therein)**.** In addition, tricin (**37)** has been reported to be an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells (Jeong et al., 2007) and colon cancer cells (Cai et al., 2004; Hudson et al., 2000), and has been considered safe enough for use in clinical studies (Verschoyle et al., 2006b).

Furthermore, 3',4',5'-trimethyltricetin (**40)** was identified, for the first time, as a wheat constituent using MS-MS analysis. It exhibited a parent ion peak in the positive mode at *m/z* 345 corresponding to [M+H]+ and two major product ions at *m/z* 315 and 255 (Fig. 2-3). Its spectral data and Rt value were identical to those obtained with an authentic reference sample. 3',4',5'-Trimethyltricetin (**40)** has recently been reported as the final enzyme reaction product of a wheat recombinant OMT catalyzing the sequential methylation of the pentahydroxyflavone, tricetin as substrate (Zhou et al., 2006). A recent review of the occurrence and distribution of tricetin methyl ethers in plants (Wollenweber, 2008) indicates that 3′,4′,5′-trimethyltricetin (**40)** is a typical constituent of grasses, and has been identified as a natural constituent in 18 graminaceous species, but not including wheat (Kaneta, 1973).

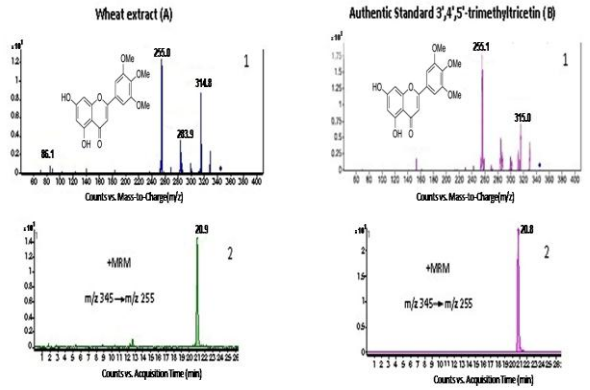


Figure 4: Identification of 3',4',5'-trimethyltricetin (40) in wheat leaves.

A, HPLC-MS (1) MS-MS for m/z 345 [M+H]+ showing 255 and 315 as major product ions, (2) MRM signals at 345 and 255 ions at Rt 20.9 min.; B, corresponding MS-MS and MRM of standards (1)

Coumarins and Simple phenolic compounds

Esculetin **(9)** and its 7-methyl derivative, scopoletin **(17)** were identified in both wheat varieties (Table 1). The use of available reference compounds enabled us to compare their Rt and MRM profiles. Under non-acclimated conditions, the amount of scopoletin **(17)** is higher than esculetin, i.e. 0.06% compared to 0.01% of total phenolic compounds, respectively. This may be explained by the possible toxicity of the vicinal hydroxyl groups present at positions 6 and 7 of the coumarin structure, which may be reduced by methylation of esculetin **(9)** to scopoletin **(17)**.

The fact that trace amounts of simple hydroxycinnamic acids were observed in the extracts of both wheat varieties (Table 2-1) corroborates with the natural occurrence of their conjugated forms as esters or glycosides (Dixon and Paiva, 1995). In contrast, two hydroxybenzoic acids, vanillic acid (**10)** and sinapic acid (**15)** were present in the free state as minor constituents.

Effect of cold acclimation on the phenolic profiles of winter and spring wheat

Wheat leaves (7-days-old) of both varieties were cold acclimated at 4°C for 0, 6, 12, 21and 42 days, and their phenolic profiles were determined by HPLC–UV methods, using quercetin as the internal standard, as described in the Experimental. During cold acclimation, there was no qualitative difference in the phenolic profiles of both varieties, but there was a significant accumulation of phenolic derivatives, reaching their highest level (2-fold increase) after 42 days of cold acclimation as compared to the corresponding non-acclimated plants (Fig. 2-4). The relative amount of phenolic compounds was consistently higher in the winter variety (Claire) than the spring variety (Bounty).

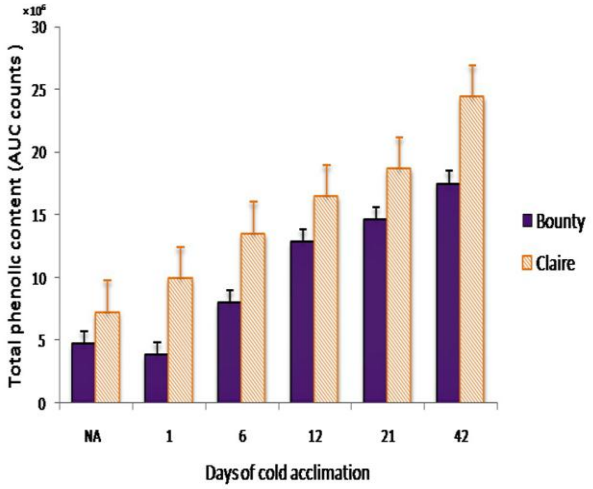


Figure 5: Total phenolic content during cold acclimation of both winter (Claire) and spring (Bounty) wheat. ***(AUC): absorption unit counts. (NA): 7-day-old non-acclimated plants.***

Both scopoletin (**17)** and esculetin (**9)** increased in their levels by 3- and 7-fold, respectively, after 21 days of

cold acclimation of both varieties. However, their contribution to the phenolic pool is still limited due to their initial low relative abundance.

The increase in phenolic compounds in the winter variety is mostly represented by the *C*-glycosides of luteolin, iso-orientin **(13)** and orientin **(12)** (*ca* 3-fold) their methylated conjugates (1.3- to 2-fold) and of apigenin, vitexin **(21)** and isovitexin **(22)** (*ca* 3-fold).

Luteolin *C*-hexosyl-*O*-(*p*-coumaroyl) hexoside **25** (Table 2-1 and Fig 2-5), which accumulated in significant amounts (10- to 15-fold) in the cold acclimated winter variety, was identified based on its mass spectrum [M+H]+ of 757.2 *m/z* and its product ions at *m/z* 757, 177, 463 and 287; where 177 is a characteristic fragment ion of *p*-coumaric acid. Moreover, its measured accurate mass was 756.1906 with only 0.6 ppm difference from the theoretical calculated value. Compound **25** reached its maximum concentration (0.2 to 0.3±0.14µM QE.g-1)2 after 21 days of cold acclimation and represented one of the major phenolic constituents, amounting to approximately 9% of the total leaf phenolics. Although its role in wheat is not clear, this luteolin derivative **(25)** was reported to be associated with the protection of a UV-tolerant rice cultivar against UV-B radiation (Markham et al., 1998).

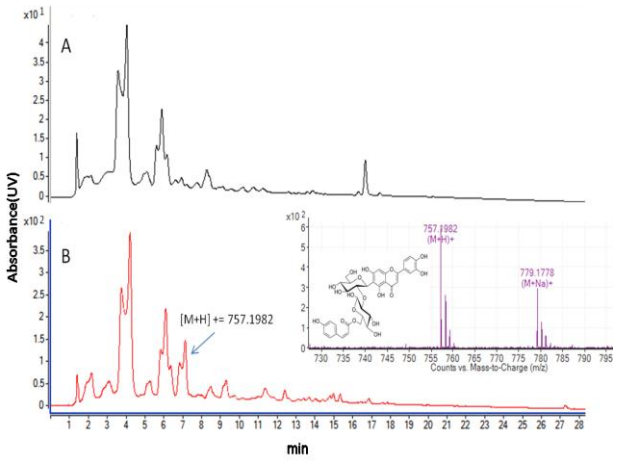


Figure 6: HPLC-UV-MS chromatograms of methanol extract of (A) non- acclimated and (B) 21-day cold acclimated Claire leaves.

Showing induction of levels of luteolin-C-hexosyl-O-(p-coumaroyl) hexoside (25) (Rt 7.2 min) corresponding to the measured accurate mass.

The fact that a number of HCAs especially compounds **4**, **5** and **31,** accumulated in wheat in significant amounts (*ca* 17- to 20-fold) in response to cold acclimation suggests a biological role in plant protection against low temperatures.

Feruloylagmatine **(5),** (Table 2-1 and Fig.2-6) was the most abundant HCA whose level was induced by cold treatment; it increased *ca* 20-fold after 21 days of cold acclimation of the winter variety. This result is in agreement with the recent finding of a 10-fold increase of feruloylagmatine **(5)** in wheat crowns exposed to low temperature (Jin and 64

Yoshida, 2000). These authors attributed its induction to its antifungal properties against the phytopathogenic fungus, *Microdochium nivale*. HCAs are synthesized in the cytosol and transported towards the cell wall, where they function as a resilient barrier against pathogen attacks (Hahlbrock and Scheel, 1989) and refs.therein), and act as stabilizers of cell membranes (Gicquiaud et al., 2002).

Therefore, it is reasonable to assume that their accumulation in wheat in significant amounts in response to low temperature functions to protect cell membranes during cold acclimation or fungal attack. This assumption deserves further study.

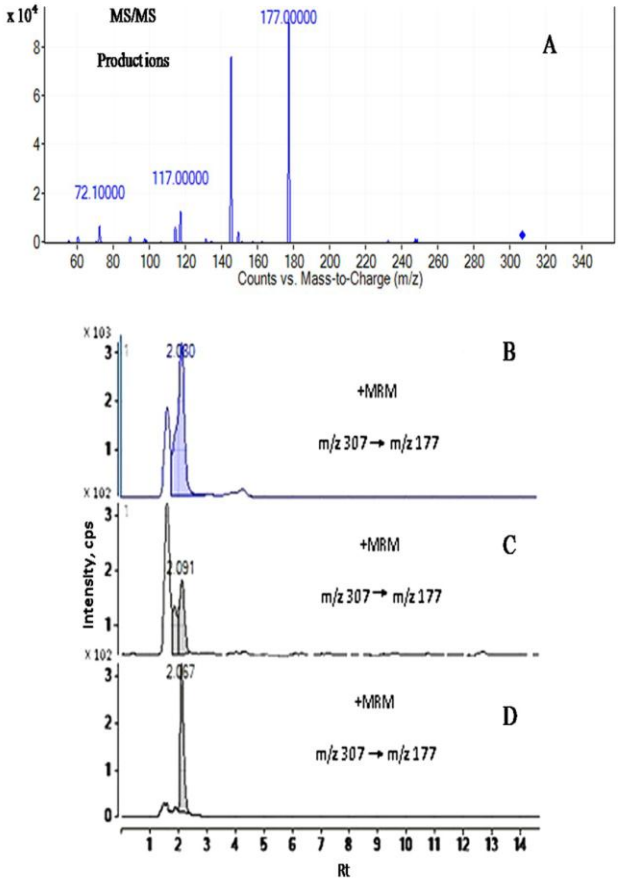


Figure 7: Identification of feruloylagmatine (5) in wheat leaves.

A, MS-MS of an authentic standard at m/z 307 [M+H]+ showing 307 and 177 as major product ions; B, shows the MRM signals 307 and 177 ions at Rt 2.07 min of 21-day cold-acclimated winter wheat leaf extract; C, corresponding non-acclimated (control) extract; D, that of reference compound.

On the other hand, cold acclimation of the spring variety Bounty also resulted in an increase in iso-orientin

derivatives **13**, **20**, **23**, **25**, and **28** (*ca*. 3-fold), similar to Claire. However, it exhibited an important increase in the HCA conjugate, dicaffeoylputrescine **31** (Table 2-1, Fig 2-7), which accounted to *ca* 15% of the total phenolic compounds after cold acclimation. The role of HCAs in plant defense against pathogens is well documented (Hahlbrock and Scheel, 1989), and was also recently reported for *Arabidopsis thaliana* (Muroi et al., 2009).

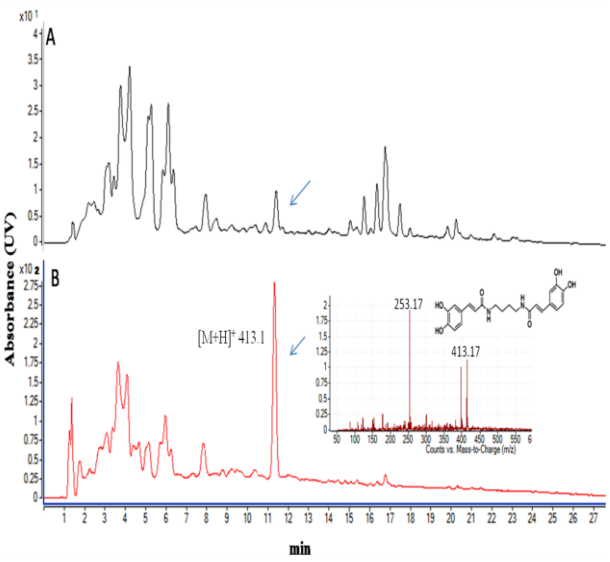


Figure 8: HPLC-UV-MS chromatogram of a methanolic extract of 6-day cold-acclimated Bounty leaves.
Showing induction of levels of compound 31, dicaffeoyl-putrescine, (Rt 11.2 min) corresponding to [M+H]+ of 413.2

The differential accumulation of two classes of phenolic compounds during cold acclimation: *C*-glycoflavones and their methylated derivatives in the winter variety, and specific HCA conjugates in the spring variety, may provisionally be explained in terms of the differential regulation of expression of the structural genes encoding chalcone synthase (CHS) and hydoxycinnamoyl-CoA:amine-*N*-hydroxycinnamoyltransferase (AHT) that are involved in the biosynthesis of flavonoids and HCA derivatives, respectively (Fig.2-8).

AHTs for both aromatic (Back et al., 2001b); (Farmer et al., 1999) and aliphatic (Negrel, 1989; Negrel et al., 1992) amines have been characterized from several plant species. Such metabolic dimorphism may be considered a valuable agricultural trait that can be applied to the engineering of wheat for increasing its cold tolerance (flavonoids) and antimicrobial constituents (HCAs), or its health promoting flavonoids.

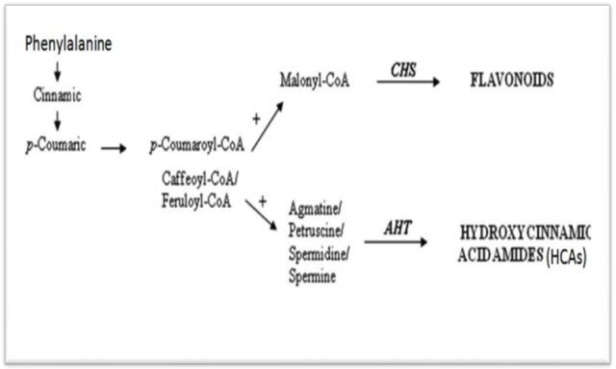


Figure 9: Proposed pathway for the regulation of flavonoid and HCAS biosynthesis.

Legends: *chalcone synthase (CHS), amine-N-hydroxycinnamoyltransferase (AHT)*

_O_-Methyltransferase activity of cold-acclimated winter wheat

The fact that the methylated derivatives **20**, **23** and **28** of luteolin (**36)** are among the major flavonoid constituents of the cold-acclimated winter variety, prompted us to investigate the methylation process by measuring *O*-methyltransferase (OMT) enzyme activity of wheat leaves. Protein extracts of 6, 12, and 21-day, cold-acclimated Claire leaves were assayed for their OMT activities against tricetin and 5-hydroxyferulic acid, as substrates. The presence of internal phenolic substrates within the crude protein extract was accounted for, by subtracting the activity of the enzyme in the absence of added external substrates. The enzyme activity with internal substrates was used as a blank value of the reaction which varied between 5 and 10% of total activity, depending on variety and cold acclimation. The OMT activity against tricetin and 5-hydroxyferulic acid (Yamamoto et al., 1987) as substrates increased by 4- to 5-fold after 12-day and 21-day cold acclimation (Fig. 2-9). The increase in OMT activity is paralleled with the observed increase of methylated phenolic compounds during cold acclimation.

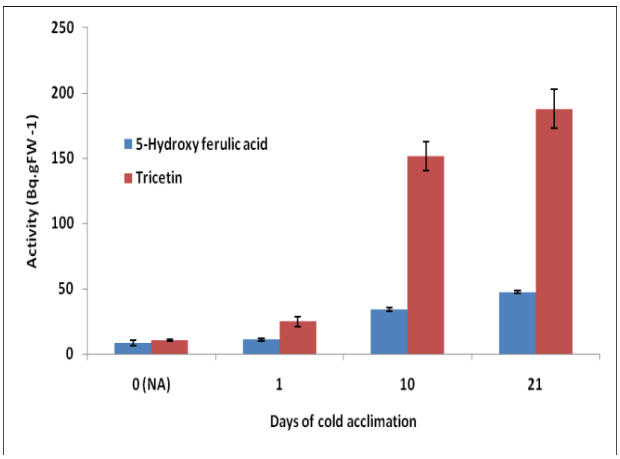


Figure 10: Changes in total methyltransferase (OMT) activities in winter wheat leaves during cold acclimation against tricetin and 5-hydroxyferulic acids as substrates.

Values represent mean ± SE from two independent experiments. (NA), 7-day-old non-acclimated plants.

Localization of phenolic derivatives in leaf apoplast fluid

Leaf apoplast is not only considered a storage cellular compartment but also an internal physiological environment of the plant where important reactions, such as intercellular signaling and cellular response to many abiotic and biotic stress stimuli, take place (Fecht-Christoffers et al., 2003; Sakurai, 1998; Sattelmacher, 2001).

This prompted us to investigate the phenolic content and profile of the apoplast fluid of wheat leaves in relation to cold acclimation. The analysis of apoplastic fluid on SDS-PAGE, exhibited a typical pattern of apoplastic proteins, and the absence of any trace of Rubisco that is usually used as indicator of cell leakage. This indicated that the apoplastic extract was not contaminated with any of the intracellular metabolites**.**

The analysis of apoplastic phenolic content suggested the presence of 12 flavonoids and 5 HCAs (Figs. 2-10 and 2-11). These compounds were observed in quantities comparable to those obtained with the total methanol extracts, and their level of accumulation during cold acclimation was consistent with that observed in total extract; since the calculated % relative abundance of each compound in the apoplast extract and in the non-hydrolysed MeOH extract were identical, as well as their fold increase after cold acclimation (Table 2-1). In addition, the peaks obtained from LC-MS analyses were sharp and symmetric, with no background contaminants as those usually observed with crude plant extracts (Fig. 2-11).

p-Coumaroylagmatine (**1**), *p*-coumaroylputrescine (**3**), feruloylputrescine (**4**), *trans*-feruloylagmatine (**5**) and *p*-coumaroyl-2-hydroxyputrescine (**7**) were identified in the apoplast fluid, together with the flavonoids 8, 13, 14, 20, 23, 25 and 28 (for iso-orientin (**13**), its methylated form iso-scoparine (**23**) and their glycoside derivatives (**8,** 70 **14, 20, 25, 28**), in addition to isovitexin (**22**) and tricin derivatives (**26**, **27, 37**, and **40**). These flavonoids were found as glycosides, containing at least one sugar moiety, except for tricin (**37**) and its methylated derivatives (**40**) that were present as aglycones. Glycosylation of phenolics increases their hydrophilicity and stability, and modifies their subcellular localization and binding properties**.** The sugar moieties are responsible for enhancing the water solubility of these compounds and thus facilitate their translocation within the cell from their site of biosynthesis to reach the apoplast (Kren and Martinkova, 2001; Wang and Hou, 2009). The hydrophobic flavonoids that represent most of the identified compounds in the apoplast extract are probably translocated to the apoplast by transmembrane protein carriers, such as ABC transporters. These transporters were found to play an important role in the translocation of isoflavone aglycone genistein into the soybean apoplast by ABC- transporter (Zhao and Dixon, 2010), an example among others.

The presence of such flavonoids and HCAs in the apoplast suggests its vital role as the first site of plant defense against abiotic stresses, such as low temperature. This finding is consitent with a recent proteomics analysis demonstrating the activation of pathogen defense enzymes (β-1,3-glucanase, peroxidase, PR4, and endochitinase) in the apoplast of rape seed (*Brassica napus* var. *napus*) infected with *Verticillium. longisporum.* (Floerl et al., 2008). It was also reported that these enzymes accumulate during cold acclimation in both wheat and rye (Griffith and Yaish, 2004).

The accumulation of both flavonoids and pathogen defense enzymes in the apoplast in response to pathogen attack and cold acclimation supports the hypothesis that the apoplast functions as the first line of defense against both biotic and abiotic stresses.

However, the nature of the interactions between the flavonoids and the defense enzymes in protecting the plant cell against these stresses deserves further investigation.

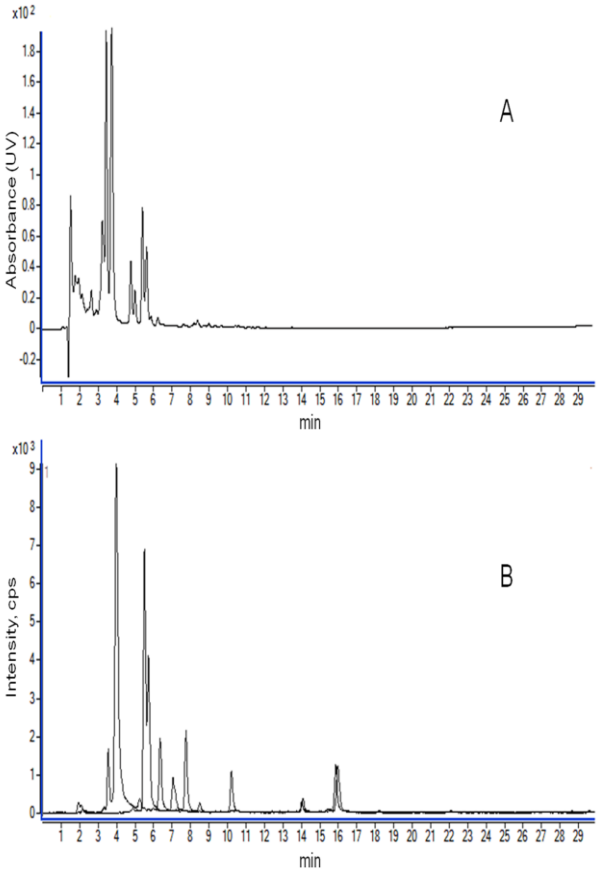
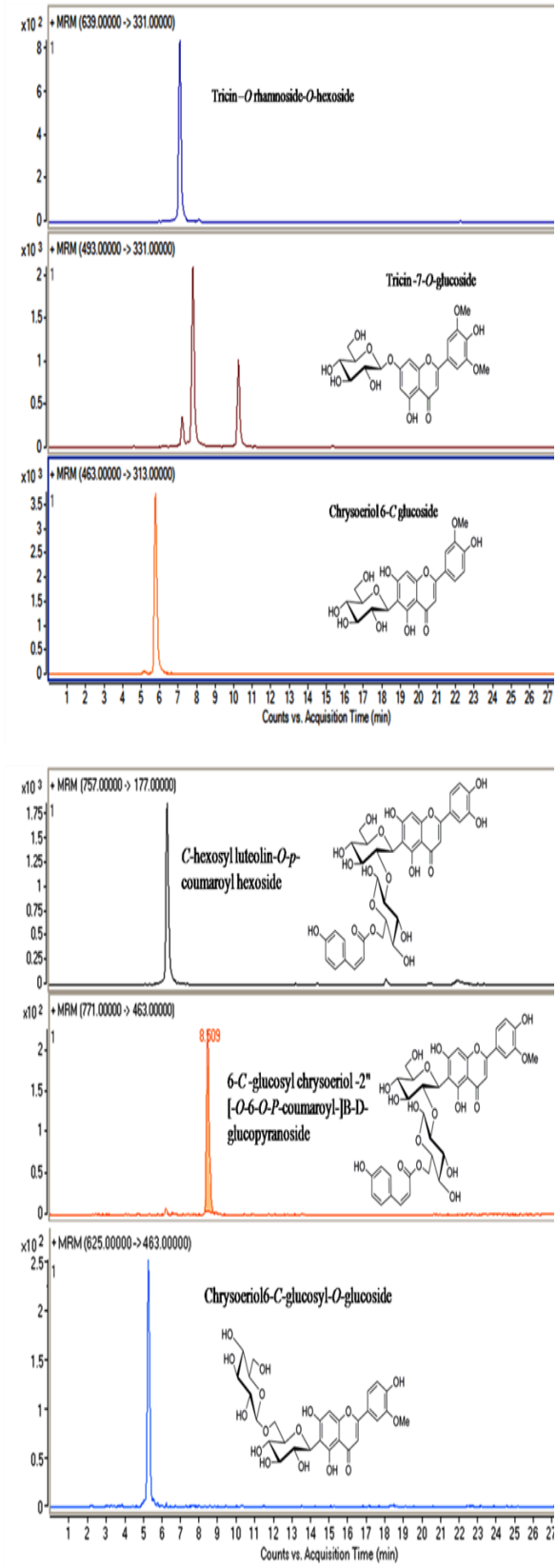


Figure 11: HPLC-UV-MS of apoplast extract from 21 day cold-acclimated winter leaves.

(A)UV-chromatogram monitored at 340nm; (B) Total ion counts (TIC)-overlaid trace chromatograms in MRM mode of the identified phenolic compounds.



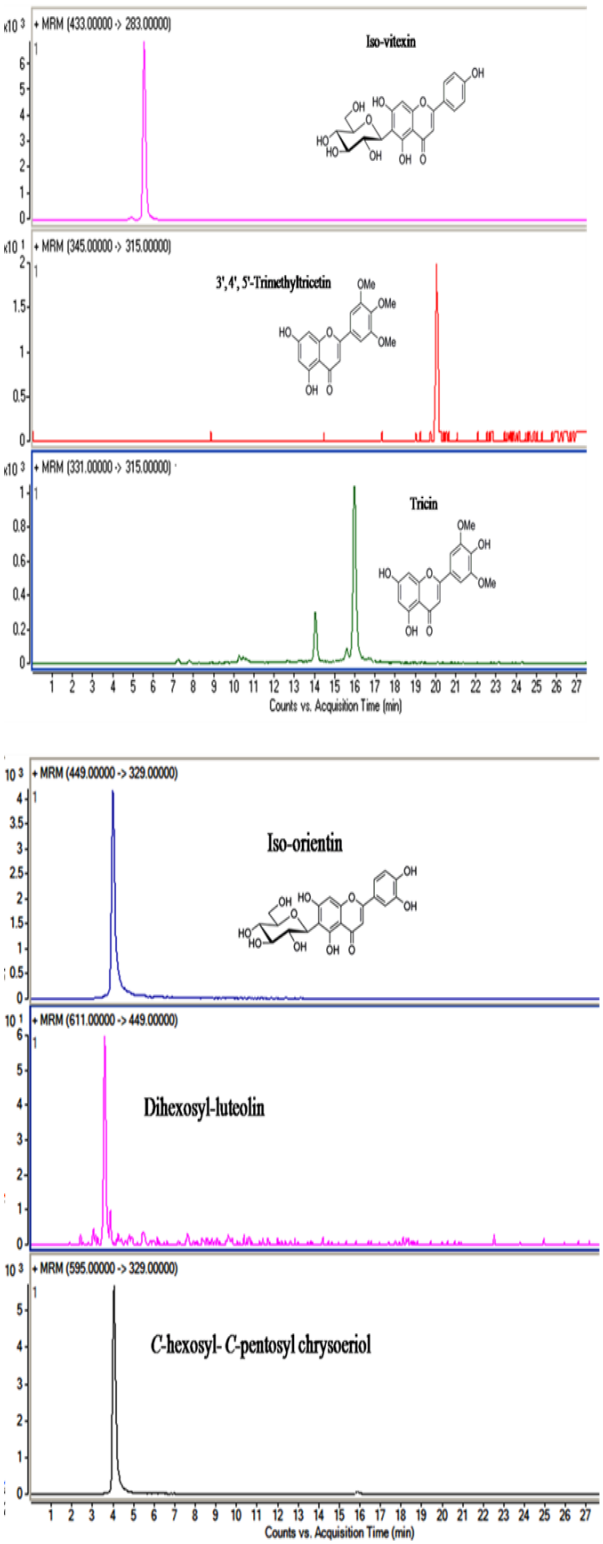


Figure 12: MRM chromatogram [MS-MS] of identified flavonoids identified in the apoplast fluid of the winter wheat Claire at 21 days of cold acclimation.

Parent ion [M+H]+ and its major ion peak fragments in the positive mode resulting from LC-MS-MS analysis, showing MRM at: 639 ->331 for tricin-O-rhamnoside-O-hexoside (26), 493-> 331 for tricin-O-malonyl hexoside (27), 463 ->313 for iso-scoparin (23), 757 ->177 for

luteolin-C-hexosyl-O-(p-coumaroyl) hexoside (25), 771 ->463, 771 ->625 for chrysoeriol-6-C -glucosyl -2"[-O-6-O-P-coumaroyl-]B-D-glucopyranoside (28), 625 -> 463 for chrysoeriol-6-C-glucosyl-O-glucoside (20), 433 ->283 for iso-vitexin (22), 345 ->255 for 3', 4', 5'-trimethyltricetin (40), 331 ->315 for tricin (37), 449 -> 329 for iso-orientin (13), 611->449, 611 ->329, for dihexosyl luteolin-(8), and595->329 for luteolin-O,C-rhamnosyl-glucosyl (14).

Table 1: Tricin content in different parts of the two wheat cultivars (Claire and Bounty).

Part of the plant	µg Tricin/g dry weight	% Tricin of total phenolic compounds
Leaves:		
Bounty	235±21.2µg/g	7-8%
Claire	253±18.3 µg/g	9-10%
Bran:		
Bounty	45±8.6µg/g	1.2 -1.5%
Claire	33±15.9 µg/g	1.8 - 2.2%
Husk:		
Bounty	408±11.3µg/g	30-35%
Claire	772±31.8µg/g	40-45%

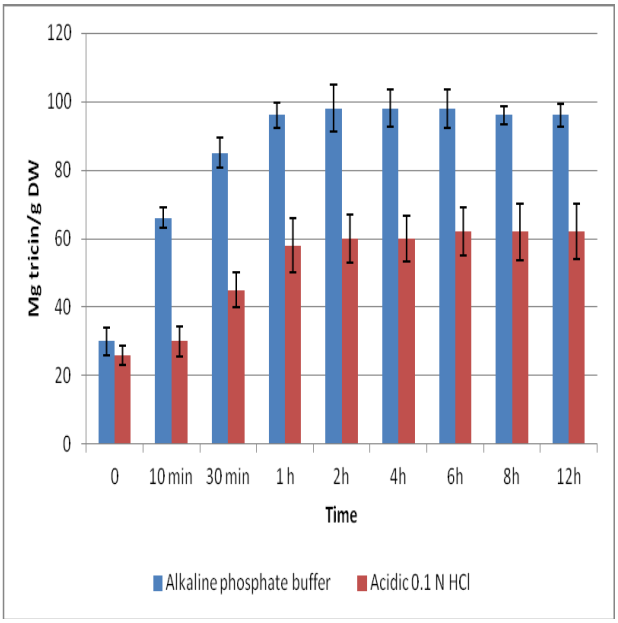
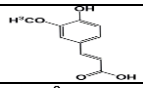
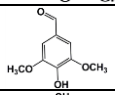
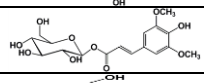
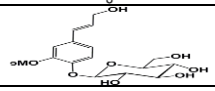
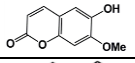
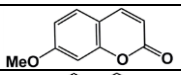
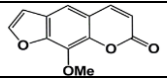
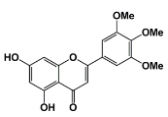
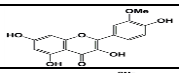
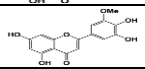
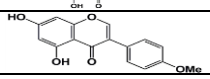


Figure 13: Solubility of tricin released from 1g dry weight Norstar husks in alkaline phosphate buffer and in acidic 0.1 N HCl, over 24h.

Table 2: The effect of selected methylated phenolic compounds on cell viability[4]

S. no.	Compound	Structure	Optimum Conc.	% Mortality		
				INS[5]	A-549[6]	NIH[7]
1	Ferulic acid		15 µM	75.1 ± 6.5 (+++)	84.9± 5.2 (++++)	17.4±9.2 (±)
2	Syring-aldehyde		5 µM	71.1±6.5 (+++)	81.7± 2.9 (++++)	13.9±4. (±)
3	Orcinol		5 µM	69.2±4.4 (++)	81.9±6.6 (++++)	15.9±6.4 (±)
4	Vanillic acid		15 µM	67.1±10.7 (++)	87.2±3.6 (++++)	18.9±5.8 (±)
5	Sinapoyl glucose		45µM	61±11.7 (++)	87.6±9.8 (++++)	14.6±7.6 (±)
6	Coniferin		30 µM	59.9± 9.1 (+)	81.4±6.8 (++++)	11.3± 7.8 (±)
7	Isosopoletin		5 µM	68.6±6.6 (++)	80.7±1.8 (++++)	15.9±5.4 (±)
8	Herniarin		30 µM	59.6±11.5 (+)	81.79±6.9 (++++)	18.9±3.3 (±)
9	Xanthotoxin		7.5 µM	53.4±9.2 (+)	82.8±10.9 (++++)	15.4±5.2 (±)
10	Trimethyl tricetin		7.5 µM	69.7±1.8 (++)	94±6 (+++++)*	15.5±7.3 (±)
			15 µM	61.1±7.2 (++)	81.2±12. (++++)	17.8±2.2 (±)
11	Isorhamnetin		30 µM	63.5±5.02 (++)	89±1.11 (++++)	23.3±8.1 (±)
12	Selgin		7.5 µM	61.2±4.9 (++)	86.9±5.4 (++++)	15.1±5.5 (±)
13	Biochanin A		30 µM	61.2±12.1 (++)	84.9±3.9 (++++)	18.5±7.2 (±)

Phenylpropanoids C1-C3 and simple phenolic compounds from compound 1 to 7, coumarins 7 to 9 and flavonoids 10-13, values represent the mean ± SE obtained from a triplicate of 3 independent experiments.
(±) 0–50%, **(+)** 51–60%, **(++)** 61–70%, **(+++)** 71–80%, **(++++)** 81–90%, **(+++++)** 91–100%

CONCLUSION

Throughout this work, we studied several aspects related to the flavone, tricin. Tricin (5,7,4'-trihyroxy-3',5'-dimethoxyflavone) is a naturally occurring flavone of relatively rare and sporadic occurrence. It is mainly found in cereal grain plants, such as rice, oat, maize, barley and wheat. Apart from being a powerful antioxidant, antimutagenic and anti-inflammatory agent, several studies have revealed the potential importance of this lipophilic flavone in cancer treatment and prevention. Tricin has been considered an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells and colon cancer cells. It is also considered safe enough for clinical studies. However, its commercial unavailability as a pure compound hinders its further experimentation.

The presence in wheat leaves of a mixture of beneficial flavonoids as tricin, iso-orientin and vitexin, values its potential use as a source of an affordable supplement of a healthy diet, which may explain the popularity of wheat leaf juice.
The use of wheat leaves (grass) is gaining ground in North America and other parts of the world. They are considered as an edible part of the plant being used as a juice (wheatgrass juice) or added to several food products. However, only few reports offer a complete profile of its phenolic content including tricin. Therefore, in (chapter 2) we investigated the phenolic profile (Phenolome) of two varieties: the winter (Claire) and spring (Bounty) varieties of wheat (*Triticum aestivum* L*)* leaves with the aim to identify, quantify and compare the most important phenolic compounds in normal and under cold conditions.

The application of LC-ESI-MS protocols, coupled with the MRM technique used, have proven to be powerful tools for the direct chemical screening of phenolic compounds in wheat leaves. They provided accurate, reproducible results, and allowed the characterization of some novel metabolites, and established the differential induction of levels of phenolic compounds in both winter and spring wheat when grown under cold acclimation conditions.

During cold acclimation iso-orientin and its *C*-glycoside derivatives followed by vitexin and iso-vitexin represented the most significant increase in phenolic derivatives of the winter variety, whereas, the accumulation of dicaffeoylputrescine was the predominant metabolite in the spring variety. Identification of the novel flavone, 3',4',5'-trimethyltricetin, as well as feruloylagmatine, by their characteristic product ion fragments, will serve as future reference sources for easy detection of both compounds in plant extracts.

Moreover, the fact that most of flavonoids (including tricin) and HCAs were identified in the apoplast compartment confirms the important role of the latter in plant defense mechanisms.

In (chapter 3) we investigated the distribution of tricin in different parts of wheat (Triticum aestivum) with the aim to designate a reliable rich source for its production. The highest amount was found in the husk of winter wheat varieties and was estimated to be 770 ± 157 µg/g dry materials. This concentration is considered the highest in any plant materials suggesting the use of winter wheat husk as a good source of tricin.

The purified wheat tricin was found to be selective potent inhibitor of two cancer cell lines of the liver and pancreas, while having no side effect on normal cells. This selectivity makes tricin a potential candidate for anticancer therapy. Thus, we describe an affordable new rich source for the chemopreventive agent tricin from a wheat waste by-product. Tricin was isolated from wheat husk that has long been considered as a waste product. The exploitation of this product for the production of tricin could change potentially its market applications.

We propose also a natural strategy for the prevention of colon cancer and liver cirrhosis through the consumption of the winter wheat-hull powder rich in both tricin and dietary fibers. This could be supplied in the form of phytonutrient-enriched food ingredient to be added to many food and bakery products, or in a suitably packaged pharmaceutical dosage form.

In (chapter 4), we wanted to further explore the selective anticancer effect of several methylated phenolic and flavonoid compounds using LDH-Spectrophotometric method to assess the viability of the cell lines. Several candidates were found to possess a remarkable antitumor activity on these malignant cell lines, such as trimethyltricetin, a tricin derivative, that exhibited a superior selective activity against human adenocarcinomic alveolar basal epithelial cells (A-549).

In (chapter 5), we tested the effects of abiotic stress factors, such as cold, drought and salt treatments, among others, on the biosynthesis and accumulation of tricin in different parts of wheat (*Triticum aestivum* L). The results show that the levels of tricin increase exponentially with wheat growth, until it reaches its maximum in the floral parts. This is associated with the increase of expression and activity of TaOMT2 suggesting an active biosynthesis of tricin in the influorescence, most probably to protect the developing seeds against any invader.

When plants are subjected to stresses that hinder growth, such as cold, salt and drought, tricin stops to accumulate. Its pathway of biosynthesis seems to be diverted to form another product, such as lignin. This suggests that tricin accumulation is associated with normal growth rate that leads to its maximum accumulation during the sensitive flowering stage and formation of seeds that need protection against biotic stresses.

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