Animal (2011), **5:4**, pp 512–521 © The Animal Consortium 2010
doi:10.1017/S1751731110002028

animal

Red clover polyphenol oxidase and lipid metabolism

G. Van Ranst[1†], M. R. F. Lee[2] and V. Fievez[1]

[1]*Department of Animal Production, Ghent University, LANUPRO, Proefhoevestraat 10, 9090 Melle, Belgium;* [2]*Institute of Biological, Environmental & Rural Sciences, Aberystwyth University, Aberystwyth SY23 3EB, Ceredigion Wales, UK*

(Received 5 April 2010; Accepted 20 August 2010; First published online 28 October 2010)

Increasing the polyunsaturated fatty acid (PUFA) composition of milk is acknowledged to be of benefit to consumer health. Despite the high PUFA content of forages, milk fat contains only about 3% of PUFA and only about 0.5% of n-3 fatty acids. This is mainly due to intensive lipid metabolism in the rumen (lipolysis and biohydrogenation) and during conservation (lipolysis and oxidation) such as drying (hay) and ensiling (silage). In red clover, polyphenol oxidase (PPO) has been suggested to protect lipids against degradation, both in the silage as well as in the rumen, leading to a higher output of PUFA in ruminant products (meat and milk). PPO mediates the oxidation of phenols and diphenols to quinones, which will readily react with nucleophilic binding sites. Such binding sites can be found on proteins, resulting in the formation of protein-bound phenols. This review summarizes the different methods that have been used to assess PPO activity in red clover, and an overview on the current understanding of PPO activity and activation in red clover. Knowledge on these aspects is of major importance to fully harness PPO's lipid-protecting role. Furthermore, we review the studies that evidence PPO-mediated lipid protection and discuss its possible importance in lab-scale silages and further in an in vitro *rumen system. It is demonstrated that high (induction of) PPO activity can lead to lower lipolysis in the silage and lower biohydrogenation in the rumen. There are three hypotheses on its working mechanism: (i) protein-bound phenols could directly bind to enzymes (e.g. lipases) as such inhibiting them; (ii) binding of quinones in and between proteins embedded in a lipid membrane (e.g. in the chloroplast) could lead to encapsulation of the lipids; (iii) direct binding of quinones to nucleophilic sites in polar lipids also could lead to protection. There is no exclusive evidence on which mechanism is most important, although there are strong indications that only lipid encapsulation in protein–phenol complexes would lead to an effective protection of lipids against ruminal biohydrogenation. From several studies it has also become apparent that the degree of PPO activation could influence the mode and degree of protection. In conclusion, this review demonstrates that protein-bound phenols and encapsulation in protein–phenol complexes, induced by PPO-mediated diphenol oxidation, could be of interest when aiming to protect lipids against pre-ruminal and ruminal degradation.*

Keywords: red clover, polyphenol oxidase, lipid metabolism, rumen, silage

Implications

Polyphenol oxidase (PPO) is an enzyme that has been found to be active in red clover. There is a whole range of recent studies exploring possibilities of using this enzyme to protect membrane lipids against degradation in the forage during storage and in the rumen of ruminants. Protection of lipids against degradation is of importance as it could help to improve the fatty acid profile of ruminant products, with respect to consumer health. This review aims to summarize studies of red clover PPO and its effect on lipid metabolism to facilitate further work in this area.

† E-mail: Gijs.VanRanst@UGent.be

Background

The feeding of red clover (*Trifolium pratense*) silage as opposed to ryegrass (*Lolium* spp.) silage to dairy cows generally results in a higher level of polyunsaturated fatty acids (PUFA) in milk per unit of PUFA intake (Table 1), which was attributed to a protective effect of red clover against rumen lipid metabolism (Lee *et al.*, 2003). This is strongly supported by the higher outflows of PUFA to the duodenum in fistulated animals (Dewhurst *et al.*, 2003b; Lee *et al.*, 2003) and from continuous culture fermenters (Loor *et al.*, 2003; Table 1). Consequently, a lower apparent biohydrogenation (the microbial saturation of dietary PUFA to more saturated products) was reported for linolenic acid (C18:3n-3) and in some cases also for linoleic acid (C18:2n-6) when offered red

Table 1 *Comparison of feeding ryegrass or red clover silages on fatty acid composition of milk (g/100 g of total fatty acid) or outflow from the rumen (g/day,* in vivo *or mg/day,* in vitro*) and apparent biohydrogenation*

	C18:3 (apparent biohydrogenation)			C18:2 (apparent biohydrogenation)			Conjugated linoleic acid (CLA)		
Reference	Grass	Red clover	Significance	Grass	Red clover	Significance	Grass	Red clover	Significance
Milk									
Vanhatalo *et al.* (2007)[1] (early)	0.41	1.34	$P \leqslant 0.05$[4]	1.24	1.80	$P \leqslant 0.05$[4]	0.50	0.50	ns
Vanhatalo *et al.* (2007)[1] (late)	0.37	0.88	$P \leqslant 0.05$[4]	1.32	1.65	$P \leqslant 0.05$[4]	0.53	0.56	ns
Van Dorland *et al.* (2008)	0.90	1.04	$P \leqslant 0.1$	1.52	1.43	ns	0.82	0.71	$P \leqslant 0.1$
Al-Mabruk *et al.* (2004)	0.48	0.92	$P \leqslant 0.001$	1.24	1.54	$P \leqslant 0.001$	0.45	0.40	$P \leqslant 0.001$
Dewhurst *et al.* (2003a)	0.48	1.51	$P \leqslant 0.001$	0.90	1.47	$P \leqslant 0.001$	0.37	0.42	$P \leqslant 0.05$
Rumen outflow									
Lee *et al.* (2003)[2]	2.69 (0.92)	11.0 (0.84)	$P \leqslant 0.05$ ($P \leqslant 0.05$)	1.44 (0.86)	4.37 (0.84)	ns (ns)	0.74	1.43	ns
Dewhurst *et al.* (2003b)[2]	4.75 (0.95)	10.4 (0.89)	$P \leqslant 0.001$ ($P \leqslant 0.001$)	11.6 (0.89)	17.0 (0.87)	$P \leqslant 0.001$ ($P \leqslant 0.1$)	1.84	3.93	$P \leqslant 0.001$
Loor *et al.* (2003)[3]	11.5 (0.98)	22.4 (0.88)	$P \leqslant 0.01$ ($P \leqslant 0.01$)	33.7 (0.94)	60.9 (0.83)	$P \leqslant 0.01$ ($P \leqslant 0.01$)	6.00	4.30	ns

[1]Vanhatalo *et al.* (2007) compared grass and red clover in an early and a late cut.
[2]Results represent fatty acid outflow to the duodenum in g/day.
[3]Results represent fatty acid outflow in mg/day from continuous culture fermenters fed; forages fed to fermenters were frozen fresh, ground while kept frozen and thawed the day before feeding.
[4]Interaction with early/late cut treatment occurred; ns = non-significant at $P > 0.05$.

clover silage compared with ryegrass silage. There are strong indications that there is a chemical mechanism at the basis of this observed reduction in biohydrogenation when red clover silages are offered compared with ryegrass silages. In this respect, phenolic compounds resulting from the enzymatic reaction of polyphenol oxidase (PPO), specifically active in red clover, have been hypothesized to be able to reduce lipid degradation in silage and further in the rumen (Lee *et al.*, 2003).

This review cumulates the latest findings on red clover PPO with regard to the factors influencing its activity, analytical/methodological aspects, activation and its effects on lipid metabolism *in silo*, *in vitro* and *in vivo* in the rumen.

Introduction

Polyphenol oxidases (PPO) are a widespread group of enzymes that include tyrosinases and catechol oxidase and are found in animals, plants and fungi (Mayer, 2006). All PPO have a dinuclear copper centre, and catalyse the insertion of molecular oxygen in the *ortho*-position to an existing hydroxyl group in phenols, resulting in an *ortho*-diphenol and its further oxidation to a quinone, although a group also shows *para*-diphenol activity. The resultant quinones will readily react with cellular nucleophiles such as certain amino acids and phenolic structures. When binding with other phenols, a melanin-like polyphenolic structure in and between proteins can be formed. This results in a brown colour, which is undesirable in fruit and vegetables (Yoruk and Marshall, 2003).

The physiological function of PPO in plants has yet to be elucidated; for example, plant defence against pathogens (Thipyapong and Steffens, 1997), the biosynthesis of floral pigments (Nakayama *et al.*, 2001) or detoxifying oxygen species in the chloroplast (Sherman *et al.*, 1995).

Measuring PPO activity

Generally, PPO activity in plant material is measured spectrophotometrically in a protein extract. Tissue is extracted, mostly with an ascorbic acid containing buffer around pH 7.0. The ascorbic acid in the extraction buffer is used to prevent PPO activity during extraction. Next, a protein purification step is performed (e.g. use of desalting column or protein precipitation using acetone) and finally activity is measured in a buffer using a synthetic substrate (4-methyl catechol is most commonly used). Increase in absorbance per unit time is a measure for the PPO activity in the protein extract and is reported as a change in optical density or unit (U) of enzyme activity. More recently, the SI unit of enzymatic activity the katal (mol/s) has been proposed and should be universally adopted when reporting PPO activity (Sullivan and Hatfield, 2006; Schmitz *et al.*, 2008; Lee *et al.*, 2010). There is a wide range of studies with regard to PPO in red clover; yet, no uniform protocol is used for PPO activity measurement, with regard to the extraction solutions or buffers and procedure, protein purification, measuring buffer, substrate and wavelength (Table 2). It should be clear

Table 2 *Extraction buffer, protein purification, measuring buffer, substrate, wavelength and unit used to measure PPO activity in four studies*

Reference	Extraction buffer	Protein purification	Measuring buffer	Substrate	Wavelength	Unit
Lee *et al.* (2006a)	McIlvaine buffer with 75 mM ascorbic acid (pH 7.0)	Desalting with bio-gel P6DG	McIlvaine buffer with 0.001 mM copper sulphate and 0.25% SDS (pH 7.0)	10 mM 4-methyl catechol	420 nm over 40 to 60 s	ΔOD/g FW per min
Van Ranst *et al.* (2009b)	0.01 M phosphate buffer with 30 mM ascorbic acid (pH 7.0)	Precipitation with 80% acetone and redissolving in phosphate buffer after centrifugation	0.01 M phosphate buffer (pH 7.0)	10 mM 4-methyl catechol	400 nm over 60 s	ΔA/mg protein per min
Polovnikova and Voskresenskaya (2008)	60 mM K-Na-phosphate buffer (pH 7.4)	Filtering	60 mM K-Na-phosphate buffer with 0.93 mM *p*-phenylenediamine (pH 7.2)	50 mM pyrocatechin	560 nm	μmol substrate oxidized/g FW per min
Sullivan and Hatfield (2006)	20 mM Tris and 100 mM ammonium acetate with 50 mM ascorbic acid (pH 7.5)	Gel filtration with sephadex G-25	McIlvaine buffer with 2-nitro-5-TNB (pH 7.0)	2 mM caffeic acid	412nm	nmol TNB/s per mg

PPO = polyphenol oxidase; OD = optical density; SDS = sodium dodecyl sulphate; FW = fresh weight; A = absorbance; TNB = thiobenzoic acid.
McIlvaine buffer is a citrate/phosphate buffer.

that activities measured with different methods are not comparable. Although this mainly concerns comparison across laboratories/experiments using different procedures, it is clear that research on this topic would benefit from a standardized methodology. Often, not the same PPO activity is measured as activation and degradation of the enzyme can vary over extraction and measuring methods. Differences in procedure could, for instance, explain the lack of activity in ryegrass when measured as described by Van Ranst *et al.* (2009a), contrary to Lee *et al.* (2006b), who reported a significant activity.

PPO activity in red clover

Differences in PPO activity between red clover cultivars has been reported (Fothergill and Rees, 2006) and was supported by our own unpublished analysis on 14 red clover cultivars that showed a 3-fold difference between minimum and maximum activities. However, PPO activity is affected to a greater extent by (generally unknown) external factors, as indicated in several studies: (i) in unpublished results of Van Ranst *et al.* the average PPO activity measured in 14 red clover cultivars after re-growth in the greenhouse was found to be about 2-fold higher compared with the PPO activity measured in the same red clover cultivars grown in the field. Furthermore, the effect of the environment (greenhouse *v.* field) differed between cultivars; (ii) Winters *et al.* (2008) found an effect of growing season as a 4-fold difference in PPO activity in red clover leaves that was measured between June and July; (iii) Fothergill and Rees (2006) also measured a seasonal effect, but in addition, they found a 5-fold higher activity in September 2005 compared with September 2004 and a 2-fold lower activity in May 2005 compared with May 2006; (iv) PPO activity was shown to be up to 5-fold higher when red clover was grown in a polluted city area compared with a relatively unpolluted area (Polovnikova and Voskresenskaya, 2008). An effect of growth stage (Polovnikova and Voskresenskaya, 2008) and re-growth duration (Lee *et al.*, 2009a) was also reported. Nevertheless, Lee *et al.* (2004) selected red clover plants (*cv. Milvus*) with consistently high or low PPO activity, in which the low PPO activity subgroup was reported to be a genotypic mutant (Winters *et al.*, 2008). This suggests that despite demonstrated effects of the environment, breeding for PPO activity in red clover is feasible.

Red clover PPO activity has been further unravelled using molecular techniques. Genetic coding for the protein in plants ranges from a single gene in the moss, *Physcomitrella patens* (Richter *et al.*, 2005) to seven genes in tomato (*Solanum lycopersicum*). Following nuclear transcription and translation the protein is translocated to the thylakoid lumen of plastid organelles (e.g. eoplasts, chloroplasts and leucoplasts). In red clover at least three genes are encoding three different PPO enzymes, namely PPO1, PPO2 and PPO3 (Sullivan *et al.*, 2004). They were found to be 87% to 90% identical on the nucleotide level. PPO1 was found to be expressed mainly in young leaves, PPO2 in flowers and petioles and PPO3 in leaves and possibly in flowers. Further studies showed that they can also differ in

activity (990, 10 and 330 nkatal mg/protein), pH optimum, stability at high temperatures and substrate specificity (Schmitz *et al.*, 2008). In a more recent study, a cluster of six PPO genes was found in red clover, comprising three variants of PPO1 (PPO1/2, PPO 1/4 and PPO1/5) and two other single-copy genes, PPO4 and PPO5, aside from the earlier detected PPO2 and PPO3 (Winters *et al.*, 2009). In-depth genetic studies of red clover PPO can not only help to establish the function of PPO in the plant, but also provide genetic markers, which could facilitate breeding.

PPO activation in red clover

In several plant species, PPO is present in a form that exhibits only low or undetectable activity, that is, a latent version. This latent PPO has been hypothesized to be due to the presence of a terminal peptide, which acts as a shield over the active site (Jimenez and Garcia-Carmona, 1996), a theory which was further supported by the work of Gerdemann *et al.* (2002). Indeed, latent and active PPO protein have been reported to differ by approximately 15 kDa in broad bean leaves (Robinson and Dry, 1992), indicating that PPO can be activated by proteolytic cleaving. Therefore, PPO can be considered to exist as a latent or pre-enzymatic form that has to be activated to elicit its full enzymatic potential, that is, the active form.

The active and total (latent + active) PPO activity present in red clover can be determined by assaying PPO activity in the absence and presence of sodium dodecyl sulphate (SDS; 0.5 to 8.75 mM) in the measuring buffer, respectively (Mazzafera and Robinson, 2000; Lee *et al.*, 2006a). SDS is a protein unfolding detergent, which has been shown in low concentrations to activate latent PPO to the active form. The action of SDS has been hypothesized to induce limited protein unfolding, which exposes the active site of the latent form (Moore and Flurkey, 1990).

Winters *et al.* (2008) showed that, on average, over the growing season about 20% of the red clover PPO was present in the active form. However, Lee *et al.* (2009a) reported that in red clover at 4 and 8 weeks of re-growth, active PPO represented 29.0% and 81.2% of total PPO present, respectively. This large variation was stated to be due to maturity of the crop and the proportion of senescent material, which significantly increased the proportion of the active enzyme. However, total PPO activity (active + latent) was 4-fold lower in the more mature forage. A higher proportion of active PPO can also be expected when red clover is exposed to pathogens and abiotic stress. This might explain the 1.5-fold higher proportion of active PPO in spongy mesophyll cells compared with palisade mesophyll cells (Lee *et al.*, 2010); as the spongy mesophyll cells are associated with the stomata, which are more likely to be subject to pathogenic attack (e.g. fungus, viral or bacteria). Hence, these cells may require higher levels of PPO as a defence response than cells that are located under the upper epidermis layer away from the stomata. Lee *et al.* (2010) also showed a 2.4-fold increase in PPO activity in both cell types when the cells were stressed through wilting and crushing the leaf.

Many induction factors of PPO have been confirmed across many different species, such as pathogen invasion (Li and Steffens, 2002), attack of herbivorous insects (Steinite *et al.*, 2004), influence of wounding (Stewart *et al.*, 2001) and stress-induced signalling compounds such as jasmonic acid (Constabel and Ryan, 1998; Gowda and Paul, 2002). The manner of induction and activation of latent PPO comprise: (i) activating the PPO genes, enhancing expression or *de novo* synthesis and (ii) changing original PPO activity through the activation of latent PPO enzyme. Activation in plastids has been shown to occur due to solubilization, interconversion (Veltman *et al.*, 1999), chemical modification such as intermolecular disulphide bridge formation, glycosylation, phenolic glucosides (Mayer and Harel, 1979), proteolytic cleavage of a pre-peptide region (Jimenez and Garcia-Carmona, 1996) and dissociation of an enzyme–inhibitor complex (Dogan *et al.*, 2002).

For red clover PPO, specifically, Lee *et al.* (2009a) correlated degree of cell damage with activation, suggested that the loss of cellular integrity further induced activation and PPO activity. Quinones, the product of oxidation of the diphenolic substrate, were also suggested to initiate PPO activation. In the presence of diphenol substrate and ascorbic acid, which prevents oxidation of diphenols to quinones, no activation of PPO was induced. However, under the same conditions, but without ascorbic acid, PPO activation occurred. Binding of quinones to the PPO enzyme was suggested to cause this effect through modifications in enzyme conformation, exposing the substrate binding site (Winters *et al.*, 2008). Activation *in situ* by the oxidized substrate is found to be a relatively fast process ($<$3 min), which can even occur during extraction (Winters *et al.*, 2005), whereas proteolytic cleavage is a rather slow process ($>$30 min; Lee *et al.*, 2009a). In healthy red clover tissue activation is prevented by sub-cellular compartmentalization of PPO, present in the chloroplast (Mayer, 2006), and its diphenolic substrates, phaselic acid and clovamide, which are thought to reside in the vacuoles (Lee *et al.*, 2009a). This explains the correlation between the degree of cell damage and PPO activation through intercellular mixing of the enzyme and substrate. *In vitro* activation by temperature treatment ($>$3 h at 65°C) has also been reported (Schmitz *et al.*, 2008) and acid/base activation was suggested by Lee *et al.* (2009a), as they measured a higher PPO activity in an extract of fresh red clover at pH lower than 5 and higher than 7.

PPO and lipid protection

PPO in red clover has been found to play a role in the reduced degradation of proteins in red clover silages (Jones *et al.*, 1995; Sullivan and Hatfield, 2006). It has also been linked with lower rates of plant lipolysis as tested *in vitro* (Lee *et al.*, 2004). This suggests that proteolysis as well as lipolysis can be inhibited by PPO activity. This review will focus on the lipid-protecting role of PPO. There are three

hypotheses of how PPO would protect lipids from lipolysis in the silage. First, quinones formed due to PPO-mediated diphenol oxidation, which can bind to amino acids and other phenols; as such, they can polymerize in and between proteins, consequently denaturing them. As enzymes, for example, lipases, are also proteins; they can be inhibited. Owing to inhibited plant lipases, a reduction in plant-mediated lipolysis can occur (Lee *et al*., 2004; Van Ranst *et al*., 2009b). Second, lipids and proteins are in close proximity embedded in a cell/organelle membrane (e.g. in the chloroplast) when quinones polymerize in and between membrane proteins, they could form a protecting envelope, protecting lipids from degradation (Lee *et al*., 2008b; Lourenço *et al*., 2008). Third, some polar lipids have potential nucleophilic binding sites, thus direct binding of quinones to lipids protects the lipids from enzymatic degradation.

Which of these three mechanisms is the most important in silages has not yet been studied directly, as protocols to measure the degree of protection through protein and lipid complexes are lacking. However, some distinction of the role of either of the mechanisms can be made based on (i) direct assessment of lipase activity and (ii) time series of fatty acid release *in silo.* Further distinction of the mechanism of lipid protection may be deemed from enzyme activation measurements, *in vitro* buffer experiments, experiments *in silo* and in the rumen, which are discussed in this section.

In vitro *studies*

Plant lipase inhibition. A first indication of the inhibition of lipases due to protein-bound phenols was reported by Van Ranst *et al.* (2009a). They showed a decrease in measured lipase activity (from 13 $\pm$ 1.4 to 7.7 $\pm$ 0.97 μmol PNPB min/g per dry matter (DM)) in association with an increase in protein-bound phenols (from 1.2 $\pm$ 0.06 to 1.8 $\pm$ 0.07 nmol tyrosine equivalent/mg protein) during wilting from ca. 300 to 500 g kg/DM. A second indication was a higher lipase activity in a protein extract of red clover after incubation for 24 h when phenols were scavenged (using polyvinylpyrrolidone) compared with the incubation in which phenols were still present (between 56.7 $\pm$ 4.24 and 19.8 $\pm$ 1.03 nmol methylumbelliferyl oleate (MBF)/min per mg DM, respectively; Van Ranst *et al*., 2006b). In a more elaborate study (Van Ranst *et al*., 2009c), in which a synthetic diphenol was added to a purified protein extract of red clover and incubated for 120 s, there was a clear correlation between measured lipase activity and the amount of protein-bound phenols (Figure 1). Furthermore, the increase in protein-bound phenols during these incubations was lower when PPO was partially inhibited using NaCN and no increase was found when PPO was almost entirely inhibited at the highest NaCN concentration. Further evidence of the inhibition of the PPO of lipase was shown with the correlation of lipase activity and protein-bound phenols in red clover that was wilted for 4 or 24 h following one of three tissue damage regimes: no damage, crushed or freezing followed by thawing (frozen/thawed (FT)). Although at higher amounts of protein-bound phenols, the link became less clear, possibly indicating that due to cell compartmentalization in the

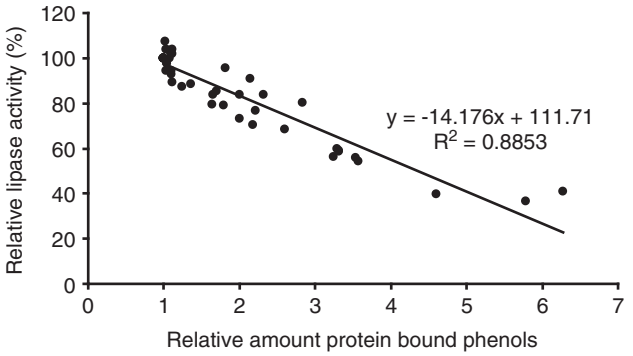

Figure 1 Relation between relative amount of bound phenols and relative lipase activity in a protein extract of red clover after incubation (120 s) with 4-methyl catechol, both were calculated as the amount or activity divided by the amount or activity present in the corresponding fresh forage (adapted from data presented in Van Ranst *et al*., 2009b).

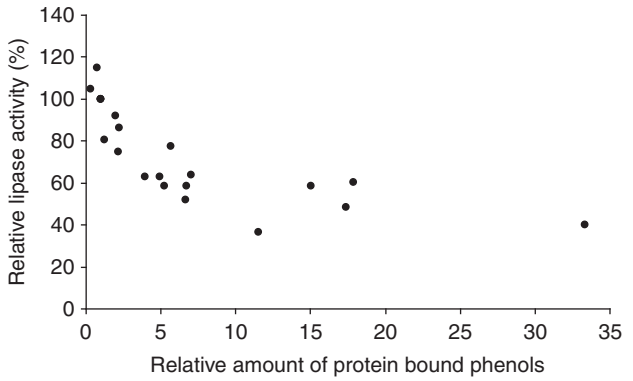

Figure 2 Relation between relative amount of bound phenols and relative lipase activity in a protein extract of undamaged, crushed or frozen/thawed red clover which was wilted for 4 or 24 h, both were calculated as the amount or activity divided by the amount or activity present in the corresponding fresh forage (adapted from data presented in Van Ranst *et al*., 2009b).

plant, the lipases that are not in contact with oxidized phenols are safe guarded from inhibition (Figure 2). Indeed, within the range of protein-bound phenols as presented in Figure 1, a negative relation between the amount of protein-bound phenols and lipase activity was observed. Although these four experiments demonstrate a link between PPO activity, protein-bound phenols and direct lipase inhibition, they do not exclude lipid protection due to protein–phenol and/or lipid–phenol complexes.

Plant-mediated lipolysis inhibition. In vitro lipolysis of forage mixed in a phosphate buffer demonstrated that a high and/or activated PPO activity results in a lower plant lipase-mediated lipolysis (Lee *et al*., 2004; Van Ranst *et al*., 2006a; Lee *et al*., 2007; Van Ranst *et al*., 2009c). In all studies a control (non-activated, low or inhibited PPO activity) was compared with treatments in which PPO activity was promoted (activation) or was high (compared with the control). In Figure 3, lipolysis of the two treatment types (control *v.* active PPO) is presented for five studies. A, B and C show

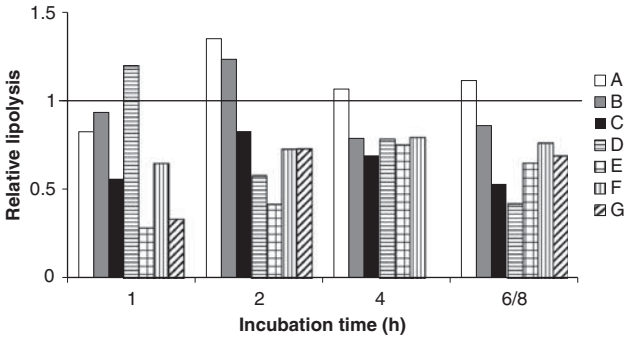

Figure 3 Lipolysis during incubation expressed relative to a lipolysis in a control during incubation for 6 to 8 h, depending on the study. A, B and C represent undamaged, crushed or frozen/thawed red clover, respectively, after 24 h wilting incubated in a buffer with ascorbic acid with fresh red clover as a control; D (Van Ranst, 2009) and E (Lee *et al*., 2004) are red clover mixed in a buffer without ascorbic acid, with ryegrass and low polyphenol oxidase red clover as a control, respectively; F (Lee *et al*., 2007) and G (Lee *et al*., 2006b) correspond with red clover and cocksfoot without ascorbic acid compared with ascorbic acid as a control.

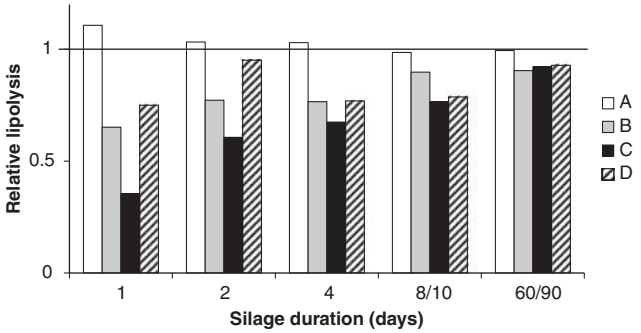

Figure 4 Relative lipolysis during ensiling of red clover; A represents lipolysis as a mean of three treatments, which did not significantly differ red clover crushed and wilted for 4 h and crushed or undamaged and wilted for 24 h (A); red clover frozen/thawed and wilted for 4 (B) or 24 h (C), expressed relative to not damaged 4 h wilted red clover (Van Ranst *et al*., 2010). D represents lipolysis in silage of red clover with normal Polyphenol oxidase (PPO) activity, expressed relative lipolysis in red clover with very low PPO activity (Lee *et al*., 2008b).

lipolysis during incubation in the presence of ascorbic acid over 24 h in which the wilted red clover was prepared under three damage regimes: not damaged (A), crushed (B) and (FT; C) (Van Ranst *et al*., 2009c). Owing to the presence of ascorbic acid, no PPO activity was possible during incubation. Therefore, only the effect of damage preparation regime on PPO activity and wilting on lipolysis was tested. Induction of PPO activity (measured as an increase in protein-bound phenols), which was FT, led to a lower lipolysis throughout the incubation. A lower degree of damaging (crushed) or wilting without damaging did not affect lipolysis, indicating the need for a high induction of PPO activity, which will include activation from latent to active PPO, to lower lipolysis.

Lipolysis has been studied during incubation of red clover compared with ryegrass (which only had a low-to-undetectable PPO activity with the analytical method of Van Ranst *et al*. (2009b) mixed in a buffer without ascorbic acid, allowing PPO activity during the incubation (D) or compared with red clover, which exhibits a low PPO activity (E) as a control. This showed that the presence of PPO activity resulted in a 25% lower lipolysis during a 6 to 8 h incubation (Figure 3). Furthermore, lipolysis during incubation of red clover with normal PPO activity (F) and cocksfoot (high PPO activity grass; *Dactylis glomerata*; G), both with and without ascorbic acid (control) demonstrated that active PPO resulted in a 25% reduction of lipolysis (Figure 3). However, from these results it cannot be concluded whether this was due to lipase inhibition or lipid protection due to protein–phenol and/or lipid–phenol complexes.

In silo *studies*

In Figure 4, it is demonstrated using data of two studies (Lee *et al*., 2008b; Van Ranst *et al*., 2010) that *in silo* lipolysis of glycerol-based cell and organelle membrane lipids can be reduced when high PPO activity or activated PPO is present. In Van Ranst *et al*. (2010), the control silage was undamaged; red clover wilted 4 h before ensiling. In this treatment, the lack of increase in protein-bound phenols after wilting suggests that PPO activation was only minor. However, longer wilting (24 h) without damaging or damaging by crushing did not have an effect on lipolysis *in silo*, as relative lipolysis did not differ from 1. Yet, when the forage was damaged by freezing and thawing, lipolysis was lower throughout ensiling. In the study by Lee *et al*. (2008b) red clover with normal and low PPO activity were ensiled after passing through a shredder.

From Figure 4, it can also be concluded that microbial lipases as opposed to plant lipases are not affected by PPO. Relative lipolysis in the study by Van Ranst *et al*. (2010) was lowest in the first days after ensiling (0.652, 0.355 and 0.750 for FT 4 h wilted, FT 24 h wilted and high *v.* low PPO activity, respectively), whereas the difference in the control largely disappeared after 60 to 90 days of ensiling (0.905, 0.921 and 0.929 for FT 4 h wilted, FT 24 h wilted and high *v.* low PPO activity, respectively). It can be assumed that during the early stages of ensiling, when silage fermentation is still at a low rate, the vast majority of lipolysis will be mediated through plant lipases. Hence, the difference in lipolysis during these first days of ensiling can be attributed to (PPO-mediated) plant lipase inhibition. However, when the fermentation rate and also bacterial activity increase, the increase in relative lipolysis can be attributed to the increasing importance of microbial lipases.

The hypothesis that PPO does not affect microbial lipases can support the observations of Van Ranst *et al*. (2009a). They ensiled red clover and ryegrass without additive, with formic acid additive (3.3 ml/g fresh) or a bicarbonate buffer additive (66.6 ml/g fresh; 0.2 M; pH 7.0). Formic acid addition restricted microbial fermentation, whereas the bicarbonate buffer extended microbial fermentation. Lipolysis in red clover, expressed relative to lipolysis in ryegrass silages, was 81.2%, 75.8% or 90.6%, for wilted undamaged silage prepared without an additive, formic acid additive or bicarbonate buffer additive, respectively. Thus, lipolysis was reduced to a higher extent when bacterial activity was inhibited by formic acid addition

Table 3 *Apparent biohydrogenation of C18:3 and C18:2 in red clover with high* v. *low PPO activity, red clover* v. *cocksfoot or fresh red clover* v. *FT red clover ensiled for 4 days (FT, 4 days) or 60 days (FT, 60 days) during 24 h incubation in rumen fluid*

	Lee *et al.* (2007)		Lee *et al.* (2008b)		Loor *et al.* (2003)[1]		Van Ranst *et al.* (2010)		
	Low PPO	High PPO	Low PPO	High PPO	Cocksfoot	Red clover	Fresh	FT 4 days	FT 60 days
C18:3	74	65*	82	72*	98	88*	97	88*	65*
C18:2	57	53*	60	55	94	83*	93	82*	66*

PPO = polyphenol oxidase; FT = frozen/thawed.
[1]Continuous culture incubation with mean retention time of solid phase of 14.3 h.
*Indicated significant ($P < 0.05$) difference from low PPO, cocksfoot and fresh, respectively.

and higher when fermentation was extended by the addition of a bicarbonate buffer.

Due to this lack of effect on microbial lipases, *in silo* lipolysis is only slightly decreased when PPO activity is present.

Effect on rumen metabolism

Table 3 summarizes four studies investigating the effect of PPO on lipid metabolism in a simulated *in vitro* rumen environment. Lee *et al.* (2007 and 2008a) and Loor *et al.* (2003) studied the biohydrogenation in incubation of red clover with high *v.* low PPO activity and red clover *v.* cocksfoot, respectively. Van Ranst *et al.* (2010) reported biohydrogenation in the incubation of fresh red clover, wilted red clover (24 h) either undamaged, FT or ensiled red clover after wilting (24 h) without and with (FT) prior damage. All the results indicate a biohydrogenation inhibiting role of PPO, as after 24 h incubation (or continuous culture incubations in the study by Loor *et al.* (2003)), biohydrogenation was consistently lower for red clover with a high *v.* a low PPO activity or red clover compared with cocksfoot. Damaging red clover by means of FT and ensiling also resulted in a decrease in ruminal biohydrogenation after 24 h incubation (Van Ranst *et al.*, 2010), indicating that damaging and ensiling red clover could also result in the protection of plant lipids.

It seems that large differences in *in silo* lipolysis, of the magnitude of fresh *v.* ensiled, are needed to generate an important difference in ruminal biohydrogenation. Indeed, the lower lipolysis in 4-day silages of the FT (50.5% $\pm$ 6.13%) compared with undamaged (70.2% $\pm$ 5.58%) red clover, assumingly due to the plant lipase-inhibiting role of protein-bound phenols, did not result in a change in biohydrogenation (87.9% $\pm$ 1.24% and 87.8% $\pm$ 0.35% of C18:3, respectively). Therefore, it can be concluded that the protection of lipids due to direct inhibition of plant lipases, can most probably not explain the higher outflow of PUFA from the rumen when red clover silages are fed. It can be assumed that PPO is inactive in the anaerobic rumen environment, as it needs oxygen to oxidize diphenols to quinones. Therefore, it is unlikely that PPO-mediated oxidation of diphenols is responsible for the inhibition of microbial lipases. Nevertheless, it has been previously demonstrated that PPO also protects plant membrane lipids from ruminal microbial lipase activity (Lee *et al.*, 2007), suggesting either the protection of lipids due to encapsulation or an alternative/additional mechanism to deactivated plant lipases.

Encapsulation in protein–phenol matrices could result in an effective (lasting) direct protection mechanism of the membrane lipids. Greater insight into the protective mechanism of PPO was highlighted in the study by Lee *et al.* (2010) who showed that membrane lipid protection incubated in rumen inoculum was apparent in macerated red clover tissue, but no protection was observed if the lipid was extracted from its cellular matrix and then incubated. This observation is consistent with a mechanism for protection of membrane lipid by PPO through entrapment of lipid within a protein–phenol complex rather than by covalent modification of lipids by PPO-generated quinones.

PPO activation rate and lipid protection

There is evidence that the mode of activation has an effect on the degree of protection of proteins and lipids. Moderate damaging (e.g. light crushing) is hypothesized to result in a moderate activation of PPO, preferably leading to binding of quinones to proteins followed by an extensive cross-linking of these quinone–protein complexes, which are (partially) undegradable by rumen microbes. Severe damaging, such as freezing and thawing, results in very rapid PPO activation, leading to binding of quinones to proteins with only limited cross-linking (Grabber, 2008).

Originally, this hypothesis was postulated by Grabber (2008) to explain the effect of the degree of damaging red clover before ensiling on the proportion of rumen undegradable protein and insoluble rumen degradable protein. They found a higher rumen undegradable protein proportion in moderately damaged red clover compared with severely damaged red clover. However, the insoluble rumen degradable protein fraction was only half that of the moderately damaged compared to the severely damaged red clover. Therefore, they hypothesized that the higher rumen undegradable protein in moderately damaged red clover silages was due to a severe complexing of phenols with proteins and further cross-linking in and between protein, and that the higher insoluble rumen degradable fraction in severely damaged red clover silages was due to the binding of quinones with proteins, without further complexing.

Applying this theory to the results of the FT and wilted red clover in the study by Van Ranst *et al.* (2009c) would suggest an inhibition of plant lipases due to the multiple binding of quinones to proteins, which leads to lower *in silo* lipolysis. However, lipids were not encapsulated due to the lack of

major cross-linking in and between proteins, explaining the lack of difference in biohydrogenation between fresh and wilted red clover. Similarly, the hypothesis could also partially explain the lack of effect of pre-conditioned red clover (FT) compared to cut red clover on the fatty acid composition of milk when offered to dairy cows (Lee *et al*., 2009b). In this study, they also concluded that the activation procedure may have altered the way PPO protected the protein and lipid as postulated by Grabber (2008). Further research is required to confirm this finding, as this could be key to fully utilizing PPO activity.

Alternative theories for a higher duodenal flow of PUFA from red clover compared with other forages

In addition, two alternative mechanisms for the protection of PUFA in the rumen during red clover feeding to those postulated for the effect of PPO should be considered, namely (i) changes in rumen microbial ecology and (ii) changes in digestion kinetics.

First, Huws *et al*. (2010) indicated that red clover silage feeding as opposed to grass silage feeding resulted in a significant change in the rumen microbial ecosystem within a 2-week period. These shifts possibly are a microbial response to compounds specific for red clover (e.g. PPO-induced protein–phenol complexes), which could affect biohydrogenating bacterial communities. Changes in the microbial population (e.g. fish oil) have already been linked with reduction and/or modification of lipolysis and biohydrogenation in some cases (Kim *et al*., 2008; Huws *et al*., 2010). However, this would not explain the lower lipolysis, which has been reported *in vitro* as well as *in silo.* Furthermore, it has been shown that these shifts did not result in an increase in the utilization of PPO-protected protein- or glycerol-based lipid (Lee *et al*., 2008a).

Second, the difference in rumen digestion kinetics between red clover and other forages could reduce residence time in the rumen and thus indirectly reduce biohydrogenation. Vanhatalo *et al*. (2007) postulated that protection of PUFA in red clover diets was a result of a combined effect of PPO protection and altered digestion kinetics with a higher rate of ruminal digestion of fibre for red clover as opposed to grass silage. This would suggest a reduction in ruminal retention time resulting in a higher proportion of dietary lipids escaping metabolism in the rumen. A similar mechanism was suggested for the elevated level of PUFA in milk observed from white clover (*Trifolium repens*) silage compared with grass silage (Dewhurst *et al*., 2003b; Van Dorland *et al*., 2008). It may also explain the lack of difference in milk PUFA, when conditioned red clover (cut/crushed and frozen and thawed), which fully activated the PPO and the cut fresh red clover with PPO activation of 29%, were fed to dairy cows, as both these treatments would likely have similar digestion kinetics (Lee *et al*., 2009b). However, a previous study has reported that the fibre content and rates of passage of red clover and grass silage were comparable (Dewhurst *et al*., 2003b) and so questions the postulation of the effect being solely related to digestion kinetics. As there are only a limited number of studies with regard to red clover digestion kinetics and they have contradictory outcomes, care should be taken when addressing this point. Nevertheless, reduced biohydrogenation has also been observed during batch *in vitro* incubations (see above) in which the outflow rate may not contribute to the observed differences.

Research needs

As the majority of the studies with regard to the effect of PPO on lipid metabolism are *in vitro* studies, it should be clear that more *in vivo* studies are needed to further confirm the link between the higher PUFA output and red clover PPO.

Fundamental research with regard to PPO's function in the plant cell, genetic expression and gene sequencing could aid in the goal of breeding for red clover with a high PPO activity. This paper focuses on PPO activity, nevertheless phenolic content and their composition can also be of importance and should be addressed in future studies.

Some creative research is still needed to further unravel the mechanism of lipid protection. Microscopic techniques to visually confirm lipid encapsulation and studying protection with and without proteins are just two examples of recent approaches (Lee *et al*., 2010). Finding conclusive evidence of this protection mechanism is crucial to further optimize and apply protection mechanisms.

Once the protection mechanism is clear, focus should be on factors influencing lipid protection through the PPO mechanism. This could be done by developing appropriate damaging and wilting regimes for red clover, but also by optimization of protection through technological treatments and industrialization (e.g. use of PPO as a natural lipid protector).

Conclusions

This review provides evidence that *in silo* lipolysis as well as ruminal lipid metabolism can be reduced when PPO activity is enhanced or high. We discussed that red clover PPO can play a direct role in the inhibition of plant lipases, through the oxidation of diphenols that bind to the lipases. However, the importance of this mechanism in the inhibition of lipolysis in the silage and further in the rumen is questionable, as this mechanism fails to protect lipids against microbial lipases, which become important during later stages of ensiling and in the rumen. Furthermore, it was also demonstrated that *in vitro* ruminal biohydrogenation is lower with silages of red clover with high PPO activity or high amounts of protein-bound phenols. Therefore, with regard to the PUFA protecting role of PPO, this led to the conclusion that lipids could be effectively protected against pre-ruminal and ruminal lipid metabolism as a consequence of the formation of protein-bound phenols and subsequent encapsulation in protein–phenol complexes.

Acknowledgements

This review paper was prepared as part of the PhD of Gijs van Ranst, which was supported by the Institute for Agriculture and Fisheries Research (ILVO), the EU-community (project Food-CT-23006-36241, Prosafebeef) and by IWT – Institute for the

Promotion of Innovation by Science and Technology in Flanders. Post-doctoral research of Gijs Van Ranst is currently supported by Flanders Food (Belgium). The authors would also like to thank all the co-workers working in the field of PPO and lipid metabolism over the last decade and all the funding streams that made this work possible, particularly BBSRC, DEFRA, ILVO and IWT.

References

Al-Mabruk RM, Beck NFG and Dewhurst RJ 2004. Effects of silage species and supplemental vitamin E on the oxidative stability of milk. Journal of Dairy Science 87, 406–412.

Constabel CP and Ryan CA 1998. A survey of wound- and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. Phytochemistry 47, 507–511.

Dewhurst RJ, Fisher WJ, Tweed JKS and Wilkins RJ 2003a. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. Journal of Dairy Science 86, 2598–2611.

Dewhurst RJ, Evans RT, Scollan ND, Moorby JM, Merry RJ and Wilkins RJ 2003b. Comparison of grass and legume silages for milk production. 2. In vivo and in sacco evaluations of rumen function. Journal of Dairy Science 86, 2612–2621.

Dogan M, Arslan O and Dogan S 2002. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. International Journal of Food Science and Technology 37, 415–423.

Fothergill M and Rees ME 2006. Seasonal differences in polyphenol oxidase activity in red clover. In Sward dynamics, N-flows and forage utilisation in legume based systems. Proceedings of the 2nd COST 852 Workshop (ed. M Wachendorf, A Helgadóttir and G Parente), pp. 141–144, Grado, Italy.

Gerdemann C, Eicken C, Galla HJ and Krebs B 2002. Comparative modeling of the latent form of a plant catechol oxidase using a molluskan hemocyanin structure. Journal of Inorganic Biochemistry 89, 155–158.

Gowda LR and Paul B 2002. Diphenol activation of the monophenolase and diphenolase activities of field bean (Dolichos lablab) polyphenol oxidase. Journal of Agricultural and Food Chemistry 50, 1608–1614.

Grabber JH 2008. Mechanical maceration divergently shifts protein degradability in condensed-tannin vs. o-quinone containing conserved forages. Crop Science 48, 804–813.

Huws SA, Lee MRF, Muetzel SM, Scott MB, Wallace RJ and Scollan ND 2010. Forage type and fish oil cause shifts in rumen bacterial diversity. FEMS Microbiology Ecology 73, 396–407.

Jimenez M and Garcia-Carmona F 1996. The effect of sodium dodecyl sulphate on polyphenol oxidase. Phytochemistry 42, 1503–1509.

Jones BA, Muck RE and Hatfield RD 1995. Red-clover extracts inhibit legume proteolysis. Journal of the Science of Food and Agriculture 67, 329–333.

Kim EJ, Huws SA, Lee MRF, Wood JD, Muetzel SM, Wallace RJ and Scollan ND 2008. Fish oil increases the duodenal flow of long chain polyunsaturated fatty acids and trans-11 18 : 1 and decreases 18 : 0 in steers via changes in the rumen bacterial community. Journal of Nutrition 138, 889–896.

Lee MRF, Parfitt LJ and Minchin FR 2006a. Lipolysis of red clover with differing polyphenol oxidase activities in batch culture. Journal of Animal Science 84 (suppl. 1), 101.

Lee MRF, Colmenero JDO, Winters AL, Scollan ND and Minchin FR 2006b. Polyphenol oxidase activity in grass and its effect on plant-mediated lipolysis and proteolysis of Dactylis glomerata (cocksfoot) in a simulated rumen environment. Journal of the Science of Food and Agriculture 86, 1503–1511.

Lee MRF, Parfitt LJ, Scollan ND and Minchin FR 2007. Lipolysis in red clover with different polyphenol oxidase activities in the presence and absence of rumen fluid. Journal of the Science of Food and Agriculture 87, 1308–1314.

Lee MRF, Tweed JKS, Scollan ND and Sullivan ML 2008a. Ruminal micro-organisms do not adapt to increase utilization of poly-phenol oxidase protected red clover protein and glycerol-based lipid. Journal of the Science of Food and Agriculture 88, 2479–2485.

Lee MRF, Scott MB, Tweed JKS, Minchin FR and Davies DR 2008b. Effects of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (Lactobacillus plantarum L54). Animal Feed Science and Technology 144, 125–136.

Lee MRF, Tweed JKS, Minchin FR and Winters AL 2009a. Red clover polyphenol oxidase: activation, activity and efficacy under grazing. Animal Feed Science and Technology 149, 250–264.

Lee MRF, Theobald VJ, Tweed JKS, Winters AL and Scollan ND 2009b. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. Journal of Dairy Science 92, 1136–1147.

Lee MRF, Tweed JKS, Cookson A and Sullivan ML 2010. Immunogold labelling to localize polyphenol oxidase (PPO) during wilting of red clover leaf tissue and the effect of removing cellular matrices on PPO protection of glycerol-based lipid in the rumen. Journal of the Science of Food and Agriculture 90, 503–510.

Lee MRF, Harris LJ, Dewhurst RJ, Merry RJ and Scollan ND 2003. The effect of clover silages on long chain fatty acid rumen transformations and digestion in beef steers. Animal Science 76, 491–501.

Lee MRF, Winters AL, Scollan ND, Dewhurst RJ, Theodorou MK and Minchin FR 2004. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. Journal of the Science of Food and Agriculture 84, 1639–1645.

Li L and Steffens JC 2002. Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta 215, 239–247.

Loor JJ, Hoover WH, Miller-Webster TK, Herbein JH and Polan E 2003. Biohydrogenation of unsaturated fatty acids in continuous culture fermenters during digestion of orchardgrass or red clover with three levels of ground corn supplementation. Journal of Animal Science 81, 1611–1627.

Lourenço M, Van Ranst G, Vlaeminck B, De Smet S and Fievez V 2008. Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. Animal Feed Science and Technology 145, 418–437.

Mayer AM 2006. Polyphenol oxidases in plants and fungi: going places? A review. Phytochemistry 67, 2318–2331.

Mayer AM and Harel E 1979. Polyphenol oxidases in plants. Phytochemistry 18, 193–215.

Mazzafera P and Robinson SP 2000. Characterization of polyphenol oxidase in coffee. Phytochemistry 55, 285–296.

Moore BM and Flurkey WH 1990. Sodium dodecyl-sulfate activation of a plant polyphenoloxidase – effect of sodium dodecyl-sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. Journal of Biological Chemistry 265, 4982–4988.

Nakayama T, Sato T, Fukui Y, Yonekura-Sakakibara K, Hayashi H, Tanaka Y, Kusumi T and Nishino T 2001. Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. FEBS Letters 499, 107–111.

Polovnikova MG and Voskresenskaya OL 2008. Activities of antioxidant system components and polyphenol oxidase in ontogeny of lawn grasses under megapolis conditions. Russian Journal of Plant Physiology 55, 699–705.

Richter H, Lieberei R and von Schwartzenberg K 2005. Identification and characterisation of a bryophyte polyphenol oxidase encoding gene from *Physcomitrella patens*. Plant Biology 7, 283–291.

Robinson SP and Dry IB 1992. Broad bean leaf polyphenol oxidase is a 60-kilodalton protein susceptible to proteolytic cleavage. Plant Physiology 99, 317–323.

Schmitz GE, Sullivan ML and Hatfield RD 2008. Three polyphenol oxidases from red clover (*Trifolium pratense*) differ in enzymatic activities and activation properties. Journal of Agricultural and Food Chemistry 56, 272–280.

Sherman TD, Le Gardeur T and Lax AR 1995. Implications of the phylogenetic distribution of polyphenol oxidase in plants. Enzymatic Browning and Its Prevention 600, 103–119.

Steinite I, Gailite A and Ievinsh G 2004. Reactive oxygen and ethylene are involved in the regulation of regurgitant-induced responses in bean plants. Journal of Plant Physiology 161, 191–196.

Stewart RJ, Sawyer BJB, Bucheli CS and Robinson SP 2001. Polyphenol oxidase is induced by chilling and wounding in pineapple. Australian Journal of Plant Physiology 28, 181–191.

Sullivan ML and Hatfield RD 2006. Polyphenol oxidase and o-diphenols inhibit postharvest proteolysis in red clover and alfalfa. Crop Science 46, 662–670.

Sullivan ML, Hatfield RD, Thoma SL and Samac DA 2004. Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in Escherichia coli and transgenic alfalfa(1[w]). Plant Physiology 136, 3234–3244.

Thipyapong P and Steffens JC 1997. Tomato polyphenol oxidase – differential response of the polyphenol oxidase F promoter to injuries and wound signals. Plant Physiology 115, 409–418.

Van Dorland HA, Kreuzer M, Leuenberger H and Wettstein HR 2008. Comparative potential of white and red clover to modify the milk fatty acid profile of cows fed ryegrass-based diets from zero-grazing and silage systems. Journal of the Science of Food and Agriculture 88, 77–85.

Van Ranst G 2009. Effect of ensiling on fatty acid composition and lipid metabolism in forages and the possible role of polyphenol oxidase. PhD, Ghent University.

Van Ranst G, De Riek J, Fievez V and Van Bockstaele E 2006a. Effects of components in red clover on plant lipase activity with possible consequences on PUFA-content of dairy products. Communications in Agricultural and Applied Biological Sciences 71, 307–310.

Van Ranst G, Lourenço M, De Riek J, Fievez V and Van Bockstaele E 2006b. Detections of potential selection factors in red clover forages to enhance silage quality with possible consequences on PUFA-content of dairy products. In XXVI EUCARPIA Fodder Crops and Amenity Grasses Section, Perugia, Italy, pp. 87–90.

Van Ranst G, Fievez V, De Riek J and Van Bockstaele E 2009a. Influence of ensiling forages at different dry matters and silage additives on lipid metabolism and fatty acid composition. Animal Feed Science and Technology 150, 62–74.

Van Ranst G, Fievez V, Vandewalle M, De Riek J and Van Bockstaele E 2009b. Influence of herbage species, cultivar and cutting date on fatty acid composition of herbage and lipid metabolism during ensiling. Grass and Forage Science 64, 196–207.

Van Ranst G, Fievez V, Vandewalle M, De Riek J and Van Bockstaele E 2009c. In vitro study of red clover polyphenol oxidase activity, activation, and effect on measured lipase activity and lipolysis. Journal of Agricultural and Food Chemistry 57, 6611–6617.

Van Ranst G, Fievez V, Vandewalle M, Van Waes C, De Riek J and Van Bockstaele E 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and in vitro rumen incubation. Animal 4, 1528–1540.

Vanhatalo A, Kuoppala K, Toivonen V and Shingfield KJ 2007. Effects of forage species and stage of maturity on bovine milk fatty acid composition. European Journal of Lipid Science and Technology 109, 856–867.

Veltman RH, Larrigaudiere C, Wichers HJ, Van Schaik ACR, Van der Plas LHW and Oosterhaven J 1999. PPO activity and polyphenol content are not limiting factors during brown cove development in pears (Pyrus communis L-cv. conference). Journal of Plant Physiology 154, 697–702.

Winters AL, Minchin FR and Morris P 2005. Substrate-dependent activation of polyphenol oxidase in red clover. XX International Grassland Congress, Dublin, Ireland.

Winters AL, Minchin FR, Michaelson-Yeates TPT, Lee MRF and Morris P 2008. Latent and active polyphenol oxidase (PPO) in red clover (Trifolium pratense) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. Journal of Agricultural and Food Chemistry 56, 2817–2824.

Winters A, Heywood S, Farrar K, Donnison I, Thomas A and Webb KJ 2009. Identification of an extensive gene cluster among a family of PPOs in Trifolium pratense L. (red clover) using a large insert BAC library. BMC Plant Biology 9, 94.

Yoruk R and Marshall MR 2003. Physicochemical properties and function of plant polyphenol oxidase: a review. Journal of Food Biochemistry 27, 361–422.