# Responses of *Lotus corniculatus* to environmental change 3: The sensitivity of phenolic accumulation to growth temperature and light intensity and effects on tissue digestibility

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| **Abstract:** | The optimum temperature and light intensity for plant growth, phenolic accumulation and tissue digestibility was determined in clonal plants of three genotypes of Lotus corniculatus cv Leo, with low, intermediate or high levels of proanthocyanidins (condensed tannins). Plants were grown from 10 o C to 30 o C, or at light intensities from 20 to 500 µm m -2 s -1 . Plants grown at 25°C had the highest relative growth rate and highest digestibility, whereas the maximum tannin content was found in plants grown at 15 o C. Approximately linear increases in leaf tannin and flavonol glycoside levels were found with increasing growth temperature in the low tannin genotype. Tannin hydroxylation increased with increasing growth temperature but decreased with increasing light intensity. The major leaf flavonols were kaempferol glycosides of which kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside were the major components. Increases in both tannin and total flavonol concentrations in leaves were linearly related to light intensity and were preceded by a specific increase in the transcript level of a non-legume type chalcone isomerase. Changes in growth temperature and light intensity therefore results in major changes in the partitioning of carbon into phenolics, which significantly affects tissue digestibility and nutritional quality with a high correlation between tannin content and leaf digestibility. |

**Responses of *Lotus corniculatus* to environmental change 3**. The sensitivity of phenolic accumulation to growth temperature and light-intensity and effects on tissue digestibility

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**Abbreviations**. BuOH - n-butanol; HCA - hydroxycinnamic acid; IVDMD - in-vitro-dry-matter-digestibility; NSC - non-structural carbohydrates; TGA - thioglycolic acid.

## Main Conclusion

Growth temperature and light intensity are major drivers of phenolic accumulation in *Lotus corniculatus* resulting in major changes in carbon partitioning which significantly affects tissue digestibility and forage quality.

## Abstract

The response of plant growth, phenolic accumulation and tissue digestibility to light and temperature was determined in clonal plants of three genotypes of *Lotus corniculatus* (birdsfoot trefoil) cv Leo, with low, intermediate or high levels of proanthocyanidins (condensed tannins).

Plants were grown from 10ºC to 30ºC, or at light intensities from 20 to 500 µm $m^{-2}$ $s^{-1}$. Plants grown at 25°C had the highest relative growth rate and highest digestibility, whereas the maximum tannin concentration was found in plants grown at 15ºC. Approximately linear increases in leaf flavonol glycoside levels were found with increasing growth temperature in the low tannin genotype. Tannin hydroxylation increased with increasing growth temperature but decreased with increasing light intensity. The major leaf flavonols were kaempferol glycosides of which kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside were the major components. Increases in both tannin and total flavonol concentrations in leaves were linearly related to light intensity and were preceded by a specific increase in the transcript level of a non-legume type chalcone isomerase. Changes in growth temperature and light intensity therefore results in major changes in the partitioning of carbon into phenolics, which significantly affects tissue digestibility and nutritional quality with a high correlation between tannin concentration and leaf digestibility.


## Introduction

It is well established that the accumulation of secondary metabolites can be affected by external abiotic factors (Dixon and Paiva 1995; Ramakrishna and Ravishankar 2013), as well as by biotic stress such as fungal attack and herbivore predation (Swain 1977; Bennett and Wallsgrove 1994; Mithöfer and Bola 2012). Response to climate change could include both increases in plant secondary metabolites to deleterious levels, or decreases in

levels resulting in reduced plant pathogen defence responses. To evaluate plant breeding strategies aimed at ameliorating such potential threats to crop productivity, herbivory or survival fitness, we need a better understanding of how changes in growth conditions affect the accumulation of plant metabolites with potentially toxic, anti-nutritional or plant defence properties.

Secondary metabolism gives rise to an enormous variety of functionally important end products with those of the polyphenol biosynthetic pathway being agronomically and economically important in a range of forage and crop plant species depending upon crop usage. These include phenylpropanoid compounds that have roles in plant support (lignin), UV protection (flavones and flavonols) and plant defence (isoflavonoid phytoalexins and condensed tannins), (Dixon and Paiva 1995). Condensed tannins can have both positive and negative effects on animal nutrition often through interactions with proteins and bacteria in the rumen (McSweeney et al. 2001). As well as contributing by-pass proteins in ruminants, they can also be antinutritional depending on their concentration and structure (reviewed by McMahon et al. 2000).

Because they reduce both digestibility and palatability condensed tannins are considered anti-nutritional at high concentrations (above 4-5% dry weight) (Barry and Duncan, 1984). In contrast, at low concentrations (2-3% dry weight) some condensed tannins are considered beneficial to ruminants as they bind to plant proteins and protect them from deamination by rumen microbes, increasing the amount of protein available for post-ruminal digestion and absorption and reducing the possibility of bloat (Barry and Manley 1984; Waghorn et al. 1987). These phenolic and polyphenolic end products are of critical value in higher plants and in forage species their tissue-specific accumulation particularly affects feed intake, digestibility and crop quality (MacAdam and Villalba 2015). However, despite considerable worldwide efforts, the full impact of condensed tannins on the nutritional value of forage has not yet provided unequivocal results. This may be partially related to the high levels of variation in the amount and structure (molecular weight and monomeric composition) as well as the tissue

distribution observed among condensed tannins from different forage and feed species when grown under different environmental conditions.

In relation to the perennial leguminous Lotus species both as crops for ruminant feed and as widely distributed species supplying food for wild herbivores, information is lacking on how environmental conditions affect both the accumulation and structure of secondary metabolites (Escaraya et al. 2012) and how this correlates with their biological activity and affects intake, digestion and animal health (Lascano et al. 2001). In *L. corniculatus* a number of phenolic end products have been identified in leaves, stems and roots. These include lignin, cell wall hydroxycinnamic acids, flavones, flavonols, induced isoflavans, anthocyanins and condensed tannins (Fig. 1).

Condensed tannin concentrations of different cultivars of both *Lotus corniculatus* and *Lotus pedunculatus* (syn: *uliginosus*), grown in different environments are highly variability, ranging of 4.7 to 8.7% dry weight in *Lotus corniculatus* and 6.3 to 11.0%, dry weight in *Lotus pedunculatus* (Kelman and Tanner 1990; Acuña et al. 2008), and while the structure of *Lotus corniculatus* tannin is suitable for improving the efficiency of protein use without reducing consumption or digestibility, the levels and structure of *Lotus pedunculatus* tannins are often suboptimal for animal production (Waghorn et al. 1990).

Increases in the accumulation of condensed tannins under controlled high temperature conditions were found in *Lotus pedunculatus* (Lees et al. 1994), but not in *L. corniculatus* (Ehike and LeGare 1993; Carter et al. 1999). Temperature effects on condensed tannins are also significantly larger in combination with other environmental factors such as drought (Anuraga, et al. 1993; Carter et al. 1999), high $CO_2$ levels (Carter et al. 1999) and soil nutrient deficiencies (Barry and Manley 1986).

Large variations in the levels of total tannins and in the ratio of free and bound tannins were also found with increasing soil nutrient and climatic stress with levels of condensed tannins above 9% dry matter being freely extractable (Barry and Manley 1986).

Low temperatures have been reported to elevate condensed tannin concentrations in *L corniculatus* (Carter et al. 1999) whilst high temperature and drought stress increased condensed tannin levels in *L uliginosus* indicating species differences in condensed tannin biosynthesis in response to temperature (Anuraga, et al. 1993).

However, few studies have determined growth and phenolic accumulation over a wide range of temperatures or light intensities, or considered the subsequent effects of environmentally induced changes in phenolics on the nutritional quality of *Lotus corniculatus*.

## Materials and methods

### Plant material

In order to test differential responses between genotypes to changes in environmental growth conditions, three specific genotypes of *L corniculatus* cv Leo were chosen for these studies which under control environmental conditions accumulated, either low (S50) intermediate (S33) or high (S41) levels of condensed tannins for this variety (Carron et al. 1994). Seed from which the three genotypes were selected were obtained from The Genetic Resource Unit, IGER Aberystwyth SY23 3EB, UK.

### Plant growth

**Temperature***:* Replicate clonally propagated plants of the three genotypes were established by crown division of mature plants into 15 cm ramets containing well-developed roots and shoots and grown in low-N compost in 13-cm pots under ambient greenhouse conditions for 6 weeks and then shoots cut back to 5 cm above soil level. Plants were then placed in 5 individual environmental growth chambers at 30, 25, 20, 15, or 10°C ± 1°C with an 18 h day at a light intensity of 450 µmol $m^{-2}$ $s^{-1}$. Other conditions such as pot size, soil type, and humidity remaining constant across environments.

The replicated whole plants (three plants per genotype per temperature), were harvested at four

to six time points during growth over a 4 to 12-week period dependent on growth temperature. Harvested shoots were weighed fresh and sub samples separated into leaf and stem, stored at -80°C, and then freeze dried and powdered for further analysis.

**Light intensity**: Replicate clonally propagated plants of the low tannin genotype S50 were established by dividing mature plants into 15 cm ramets containing well-developed roots and shoots. Plants were grown under greenhouse conditions as above, for 6 weeks and then shoots cut back to 5 cm above soil level and regrown at 20°C 16h day for 4 weeks either at different light intensities of 20, 80, 200 and 500 µmol $m^{-2}$ $s^{-1}$ , or regrown for 4 weeks at 20°C 16h day at 50 µmol $m^{-2}$ $s^{-1}$ and then the light intensity increased to 500 µmol $m^{-2}$ $s^{-1}$. Leaves from one stem of each plant were harvested over the following 12 h for *chi* transcript levels and over the following 5 days for flavonoid determinations.

**Determination of condensed tannins**

Total condensed tannins were determined as the sum of extractable (acetone soluble) and bound fractions on duplicate 20-30mg samples of freeze-dried leaves and stems using a modification of the butanol-HCl (BuOH/HCl) method outlined by Terrill et al. (1992). Samples were mixed and extracted with 4ml of 70% aqueous acetone and 2ml diethyl ether, vortex-mixed for 2 min then centrifuged at 980x*g* for 5min resulting in three phases. The upper solvent phase, containing chlorophyll pigments and lipids, was discarded, and the clear aqueous phase containing acetone-soluble condensed tannins was decanted and retained. The residue containing bound condensed tannins was then re-extracted as above. The combined aqueous phases were concentrated, adjusted to 2.5ml with distilled water and 0.5-ml aliquots hydrolysed in 3.5ml of BuOH:HCl (95:5v/v) for 1h at 100°C, followed by rapid cooling. The residue was dried in a stream of air to remove traces of solvent and directly hydrolysed in 4ml of BuOH:HCl. The hydrolysates were then scanned between 400 and 700nm by visible

spectrophotometry and the condensed tannin concentration calculated from the peak height at 550nm using an E1% 550 value of 150, derived from a standard curve of *L corniculatus* condensed tannin extracted from shoot tissue by the method of Terrill et al. (1992) followed by purification on Sephadex LH20 according to Foo and Porter (1981). Total condensed tannin in roots was determined by direct hydrolysis of powdered tissue with BuOH:HCl.

**Determination of the anthocyanidin composition of condensed tannins**

Anthocyanidins were determined as described by Bavage et al. (1997). Anthocyanidins derived from butanol-HCl hydrolysis of tannin extracts (1 mL) were evaporated to dryness in a dry heating block at 40°C under a stream of N2, and immediately re-dissolved in 50 µl methanol + 1% v/v HCl. Anthocyanidins were separated by high-performance liquid chromatography on an 8 X 10 cm µNova-Pack C18 column (Waters Inc), eluted with 5% aqueous acetic acid (A) and methanol (B), in a linear gradient of 30-100% B in 20 min and detected with a photodiode array (Waters 996), monitored at 525 nm and spectra collected between 480- 580 nm. Peaks were identified by comparison of their retention time and absorption spectra with authentic cyanidin, delphinidin, pelargonidin, fisetinidin, robinetinidin, luteolinidin and apigeninidin (Apin Chemicals, UK.). As the anthocyanidins have different wavelength absorption maximum, but similar extinction coefficients, correction factors were determined from standards and used to correct for the difference in peak area at 525 nm where cyanidin (λ maximum = 525 nm) = 1.000, delphinidin (λ maximum = 535 nm) = 1.044, and pelargonidin (λ maximum = 517 nm) = 1.076.

**Determination of flavonoids**

Flavonoids were determined using a modified version of the method described by Robbins et al. 1998. Freeze-dried samples (200 mg) were re-hydrated in 2 mL distilled $H_2O$ for 1 hour then ground using a pestle and mortar in 5 mL 70% aqueous methanol and transferred to 12

mL screw cap boiling tubes with a further 5 mL of aqueous 70% methanol. Tubes were vortexed for 3 min at 1800 rpm and centrifuged at 2500 rpm for 5 min. The pellets were further extracted with 10 mL 70 % aqueous methanol and twice with 100 % methanol. The methanol extracts were pooled, filtered into 50 mL round bottom flasks and the pellets saved for subsequent lignin analysis. Methanol was removed from the extracts under vacuum at 50$^{o}$C on a rotary evaporator and then adjusted to 50 mL with distilled $H_2O$, and 10 mL concentrated on an activated C18 Sep-Pak column (500 mg) [Waters]. Bound flavonoids for HPLC analysis were eluted with 4 mL 100% methanol. Alkali hydrolysis was carried out on 1 mL of extract with 1M NaOH at 100$^{o}$C for 1 h. The sample was cooled, diluted to 5 mL, and adjusted to between pH 6 and pH 7 with 0.1 M HCl. The sample was concentrated onto an activated C18 Sep- Pak column and the hydrolysed flavonoids eluted with 4mL of MeOH. Flavonoid profiles were obtained by reverse phase HPLC on a µNovapak C18 RCM cartridge (Waters) initially on a linear MeOH:acetic acid (5 %) gradient from 0-100% MeOH at a flow rate of 2 mL/min for 50 min, and subsequently on a 25-75% gradient for 25 min. Eluting peaks were monitored at 340nm using a diode array detector (model 996, Waters), and the spectra were recorded between 240nm and 400nm. Flavonoid glycosides and aglycones were identified by their UV/Vis spectra and retention times by comparison with aglycone standards (Sigma-Aldrich and Apin Chemicals, Ltd, Oxford UK) and with results of flavonoid analysis previously obtained (Robbins et al. 1998) and by comparison with relative retention times and spectral properties of *Lotus japonicus* leaf flavonoids (Suzuki et al. 2008). Total hydroxycinnamic acid (HCA) esters were calculated as p-coumaric acid equivalents as acid hydrolysis of extracts gave p-coumaric acid as the only HCA product.

**Determination of non-structural carbohydrates (NSC)**

Total sugars and sucrose were determined with anthrone reagent using a modified micro method based on that of Van Handell (1968) as described in detail in Carter et al 1999. Starch

determinations were carried out on the dried pellets after sugar extraction using the glucose oxidase and peroxidase method (Trinder glucose analysis kit [Sigma]) after amyloglucosidase treatment. The absorption of the resulting coloured quinoneimine dye complex was determined by spectroscopy at 510nm and compared to a glucose standard curve, as described in detail in Carter et al 1999)

**Lignin determinations**

Thioglycolic acid soluble lignin (TGA lignin) levels were determined using a modified version of the method described by Whitmore (1978). Freeze-dried samples (200 mg) were re-hydrated in 2 mL distilled $H_2O$ for one hour and then repeatedly extracted with methanol and acetone until the pellets were colourless. The extracts were discarded and the pellets dried using a sample concentrator and then treated with 10 % thioglycolic acid in 2 M HCl (3 mL/50 mg of sample) at 100$^{o}$C for 4 h. The samples were cooled and centrifuged for 5 min at 980x*g* and the supernatant discarded. The pellets were then washed with 2 mL distilled water and centrifuged again and the supernatants discarded. The pellets were then treated with 2mL 1M NaOH and vortexed for 2 h before centrifugation (10 min at 980x*g*). The supernatant was collected and the pellets were washed with a further 2mL 1M NaOH and centrifuged again for 10 min. The supernatant was collected and the extracts pooled. Concentrated HCl (1.2 mL) was added to the pooled supernatants and cooled at 4$^{o}$C for 1 h to precipitate the acid insoluble TGA-lignin complex. Samples were centrifuged for 5 min at 980x*g*, the supernatant discarded and the pellets redissolved in 5 mL 0.5 M NaOH. The TGA-lignin content of the samples was determined using a PV8700 series/UV/Vis spectrophotometer (Philips Scientific and Analytical Equipment) at 335 nm, and the TGA-lignin content was calculated from a standard curve of purified *L. corniculatus* TGA lignin extracted in bulk from *Agrobacterium rhizogenes* transformed "hairy" root cultures of *L. corniculatus*.

**Determination of tissue digestibility**

The effect of the different growth conditions on the digestibility of leaves and stems was estimated using two different methods that measure either the final end point of digestion or the initial rate at which the tissues are digested.

(i) The two-stage in vitro pepsin-cellulase solubility technique of Jones and Hayward (1973), determines the final end point in-vitro-dry-matter-digestibility (IVDMD), defined as the amount of biomass remaining at the end point of 48 h digestion of 1.0 g dry weight of powdered whole tissue. (Based on in-house reference samples included in each analysis, the RSD of the method was 0.1904)

(ii) The in vitro gas production technique of Theodorou et al. (1994), determines the initial rates of digestion under more realistic environmental conditions of the rumen, and uses a pressure transducer to quantify the increase in head-space gas pressure (and thus the gas volume) in closed batch cultures inoculated with rumen micro-organisms. The batch cultures contained 0.5g freeze dried powered tissue in 90mL of bicarbonate buffered medium and were inoculated with rumen micro-organisms in 10mL of clarified rumen fluid and incubated at 39ºC. Rumen fluid was collected as grab samples of rumen digesta from fistulated wethers fed grass hay, taken before the morning feed and transported to the laboratory in a prewarmed (39 ◦C) vacuum flask. Initial rates of digestion were calculated from the kinetics of gas evolution over the first 6h of incubation from 8-h batch fermentations.

**Transcript analysis**: ***Real-time PCR of chalcone isomerase gene family members.***

Transcript analysis methods were as described in detail by Lanot (2004). Leaf material was harvested from three plants grown for 4 weeks at 50 µmol $m^{-2}$ $s^{-1}$ over a 12 h period following an increase in light intensity to 500 µmol $m^{-2}$ $s^{-1}$ and three RNA extractions were carried out from the pooled plant material and cDNA synthesised. Two real time PCR reactions were performed per cDNA sample per gene and three PCR per gene amplified. The control value

corresponded to plant material harvested at t=0 before increasing the light intensity (three RNA extractions and three real-time PCR reactions/gene/ cDNA). The expression of three *chi* gene family members was compared to expression of β- actin using the fluorescent intercalating dye SYBR-Green to monitor RT-PCR products on line with the Bio-Rad iCycler iQ detection system. The expression of each gene was expressed as threshold cycles (Ct) and the Relative Expression Software Tool (REST©) was used to calculate the relative expression ratio.

The samples were statistically compared by a Pair wise Fixed Reallocation Randomisation Test (http://www.wzw.tum.de/gene-quantification/rest.html) as described in detail by Lanot (2004).

## Results

### The effects of temperature on growth and tannin accumulation

Plants were grown at constant temperatures from 10ºC to 30ºC and harvested four to five times over their growth period to flowering. Within the temperature range the mean maximum growth rate, when expressed either as biomass yield (g $day^{-1}$) or stem extension (cm $day^{-1}$), of the three genotypes was greatest at 25°C and slower below and above this temperature (Fig. 2 a,b), and with a lower leaf stem ratio at higher growth temperatures (Fig. 2c). However, the mean maximum tannin concentration of leaves and stems of the three genotypes was found at 15ºC (Fig. 2a b). The growth rate and tannin accumulation of the three individual, low, intermediate and high tannin genotypes are shown in Fig. S1, and the kinetics of leaf tannin accumulation in leaves and stems of the high and low tannin genotypes are shown in Figs. S2 and S3.

Growth temperature was also found to affect condensed tannin solubility in 70% aqueous acetone, with tannin solubility ranging from 35% to 75% in leaves and from 35% to 60% in stems of the high and low tannin genotypes respectively (Fig. 3).

The extent of hydroxylation of condensed tannins in leaves of both high and low tannin genotypes, was also found to be modified by growth temperature with the mean procyanidin

to prodelphinidin (PC: PD) ratio across the growth period, decreasing from 3.1 and 2.8 at 10°C to 1.8 and 1.9 at 30°C in the low and high tannin genotypes respectively (Fig. 4), indicating an increase in tannin hydroxylation with increasing growth temperature for both low and high tannin genotypes.

**Effects of growth temperature on carbohydrate accumulation**

Levels of starch (Fig 5a) and both sucrose (Fig 5b) and the reducing sugars glucose + fructose (Fig 5c) in leaves, stems and roots of the high tannin genotype were found to be up to 6-fold higher in plants grown at 10°C than in plants grown at 25°C, except for sucrose levels in stems and roots which were less affected (Fig. 5b). Most significantly in terms of carbohydrate reserves in roots available for utilization in shoot regrowth following defoliation was the 4-fold difference in root starch (Fig 5a) and reducing sugars (Fig 5c) in plants grown at 10°C compared with roots of plants grown at 25°C.

**Effects of growth temperature on flavonoid accumulation**

Flavonol glycoside levels were found to be highest in both genotypes when plants were grown at 10°C and lowest when grown at 30°C, with a light intensity of 450 µmol $m^{-2}$ $s^{-1}$ and a 18h day, with an almost linear, negative relationship between flavonol accumulation and growth temperature increase for both genotypes (Fig. 6). The kinetics of leaf flavonoid accumulation in the high and low tannin genotypes at different temperatures is shown in Fig. S4a,b.

The five most predominant flavonols accumulated in leaves were kaempferol-3-glucoside, kaempferol-3-rhamnoside, kaempferol-3-rhamnoside-7-glucoside, kaempferol-3,7-dirhamnoside and kaempferol-7-rhamnoside, of which kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside were the major components. The high tannin genotype S41 accumulated 10-30% more flavonoids at lower growth temperatures (10-20°C) (Fig. 6a) than the low tannin S50 genotype (Fig 6b), but at higher growth temperatures (25-30°C), the low

tannin genotype accumulated 30% more flavonoids than the high tannin genotype (Fig. 6a,b). Only minor amounts of the flavonol aglycone kaempferol were detected, but on hydrolysis of extracts, this was found to be the major base flavonol in the leaves.

**Effects of growth temperature on lignin solubility**

Less TGA lignin was found to be extracted from leaves, stems and roots of the high tannin genotype grown at 10ºC than at 25ºC, (in leaves by 14%, in stems by 28% and in roots by 50%) (Fig. 7).

**Effects of growth temperature on tissue digestibility**

Maximum endpoint digestion (as determined by IVDMD), and initial rates of digestion,( as determined by initial rates of gas evolution), of leaves were found from plants grown at 25-30ºC (Fig. 8b), but was less pronounced in stems, and contrasted with maximum mean tannin levels in leaves and stems, of the three tannin genotypes, of plants grown at 15ºC (Fig 8a) . The relationship between the levels of tannin accumulation and tissue digestibility in leaves and stems of the three individual tannin genotypes grown at different temperatures is shown in Fig. S 5a-c.

**Effects of light intensity on phenolic accumulation**

When established clonal plants of the low tannin genotype S50 were defoliated to 5cm and regrown at 20ºC for 4 weeks on a 16/8h day/night cycle, flavonoid concentrations in leaves were found to be nearly linearly related to light intensity (Fig. 9a), whereas total hydroxycinnamic acid ester accumulation was maximal at low light intensities (Fig. 9a), and tannin hydroxylation decreased with increasing light intensity (Fig. 9b).

Analysis of HPLC profiles and UV/Visible spectra of soluble phenolic extracts (Fig. 10) showed that leaves mainly accumulated kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside with smaller amounts of kaempferol-7-rhamnoside, kaempferol-7-rhamnoside-

3-glucoside, and kaempferol-3-rhamnoside, and three unidentified kaempferol glycosides (Fig. 10). Total flavonoids were calculated as kaempferol-3-0-glucoside equivalents as acid hydrolysis gave kaempferol as the only product. Six hydroxycinnamic acid esters were also identified as esters of p-coumaric acid as this was the only product following acid hydrolysis. As with condensed tannins, (Fig. 9a) increases in total flavonol concentrations in leaves was found to be linearly related to light intensity (Fig 11a). Flavonoid accumulation rapidly increased within one to three days by increasing the light intensity from 50 µmol $m^{-2}$ $s^{-1}$ to 500 µmol $m^{-2}$ $s^{-1}$ (Fig 11b) and was preceded by a specific increase in the transcript level of the non-legume type *chi* transcript (*chi2*) when normalised to β-actin, while no increase in expression was observed for the legume type *chi* genes (*chi1* and *chi3*) post illumination (Fig 11C). The normalised expression of the *chi* genes in all samples were significantly different than the expression at t=0 ($p<0.05$) (except for *chi3* at 1h and *chi2*, at 6 and 12h).

## Discussion

The aim of this work was to evaluate the sensitivity of phenolic accumulation in the vegetative tissue of the perennial forage legume *Lotus corniculutus* to growth temperature and light intensity and to determine the extent to which this affects its nutritional quality. Currently there are only a few reports that have evaluated the effects of environmental change on the accumulation of secondary compounds in crop plants and determined how they affect nutritional quality.

### Condensed tannin accumulation.

While some studies have investigated the effects of environmental stress on the accumulation of condensed tannins in *L. corniculatus*, they have focused either on the effects over a narrow range of temperature (Anuraga et al.1993; Lees et al. 1994; Carter et al. 1999), and have not

considered the subsequent effects on the flux of carbon into other secondary pathway end products and there is currently a paucity of information on the effect of growth temperature or light intensity on flavonoid accumulation or on lignin solubility in *L. corniculatus.*

The effect of temperature on plant growth was similar to that previously found by Kunelius and Clark (1970) who found that the optimum root temperature for symbiotic nitrogen fixation and growth of birdsfoot trefoil was from 18 to 24ºC, with plants grown at 9ºC being up to 45% of those at 24ºC and with growth depression at 36ºC.

While maximum growth rates were found at 25ºC, maximum condensed tannin concentrations in both leaves and stems, were found at 15ºC. Lower growth temperatures have previously been reported to elevate condensed tannins in *Lotus corniculatus* (Anuraga et al. 1993; Carter et al. 1999) and to decrease at high temperature (Ehike and LeGare 1993; Carter et al. 1999), as found in this study, while high temperatures and drought stress increased condensed tannin levels in *Lotus uliginosus* (Anuraga et al. 1993; Lees et al 1994). indicating species differences within the Lotus genus in condensed tannin biosynthesis in response to growth temperature. Moreover, both plant regrowth and condensed tannin levels were severely reduced after plants were defoliated and regrown at 30ºC (Lees et al 1994). However, Briggs and Schulz (1990), found that contrary to expectations, investment in condensed tannin accumulation in *Lotus corniculatus* was not at the expense of plant growth, but was positively correlated with shoot size.

The general trend of increased tannin solubility in high tannin leaves is consistent with findings, particularly in species of low protein concentration (Barahona et al. 2003). Large variations in the levels of total tannins and in the ratio of free and bound tannins were also found in other Lotus species with increasing climatic stress with levels of condensed tannins above 9% dry matter being freely extractable (Barry and Manley 1986), and not bound to protein or fibre on cell disruption. The proportion of soluble and bound tannin fractions of *L corniculatus* have also been found to be affected by leaf wilting, ensiling and pelleting with a lower portion of

soluble and higher proportion of protein-bound tannins with an emphasise that bound tannins should not be ignored since this can protect protein from ruminal degradation (Girard et al. 2008).

Condensed tannin concentrations in leaves were found to increase linearly with increasing light intensity up to 500 µmol $m^{-2}$ $s^{-1}$, and tannin hydroxylation decreased with increasing light intensity up to 200 µmol $m^{-2}$ $s^{-1}$. Light intensity has previously been shown to increase tannin accumulation in *L corniculatus* (Paolocci et al. 1999), and specifically in both S50 and S41 genotypes, as well as in S50 plants up and down regulated in tannin accumulation by expression of the maize *Sn* transcription factor (Paolocci et al 2005). Furthermore, they also showed that both the low S50 and high S41 tannin genotypes maintained a linear relationship between the number of tannin containing cells in leaves and light intensity up to 1000 µmol $m^{-2}$ $s^{-1}$

**Flavonoid accumulation**

Total flavonoid concentrations of up to 20% dry weight in the leaves and 3% in stems are major pools for carbon allocation in *Lotus*. Growing *Lotus* at a temperature below that required for optimum growth, resulted in higher concentrations of total flavonols in both leaf and stem tissues largely due to significant increases in kaempferol-3-glucoside and kaempferol-3,7-dirhamnoside.

Flavonoid concentrations in leaves were also found to increase linearly with increasing light intensity up to 500 µmol $m^{-2}$ $s^{-1}$, whereas total hydroxycinnamic acid ester accumulation was maximal at low light intensities. Leaf flavonoids were also rapidly induced and were maximal after 2-3 days of exposure of low light adapted plants to a high light intensity. Light intensity has previously been shown to increase tannin accumulation in *L corniculatus* (Paolocci et al. 1999), and specifically in both S50 and S41 genotypes, as well as in S50 plants up and down regulated in tannin accumulation by expression of the maize *Sn* transcription factor (Paolocci et al 2005). Furthermore, they also showed that both the low S50 and high S41 tannin genotypes

maintained a linear relationship between the number of tannin containing cells in leaves and light intensity up to 1000 µmol $m^{-2}$ $s^{-1}$

Correlation analysis showed a positive correlation between condensed tannin and flavonol accumulation in *L. corniculatus* shoots, which strongly suggests that the condensed tannin and flavonol biosynthetic pathways are co-regulated in shoot tissues and particularly in the leaves, and are affected by the same environmental factors. Probably the same environmental factors result in the induction of common transcription factors whose target genes are involved in both flavonol and condensed tannin biosynthesis (Robbins et al. 2002, Paolocci et al. 2005; Zhao et al. 2010), although it is also possible that transcription factors, which are unique to either flavonol or condensed tannin biosynthesis, are activated by the same environmental variables. Although the intensity of full natural daylight can be up to 2000 µmol $m^{-2}$ $s^{-1}$, and conclusions from the lower maximum light intensities used in these experiments may not apply to higher levels of irradiance, field measurements of the photosynthetic light-response curves for *L. corniculatus* in two sites were found to saturated at irradiances of ~650 µmol (photon) $m^{-2}$ $s^{-1}$, with saturation at higher irradiances normally being reported only for sun-adapted, early successional species (Kostopoulou and Karatassiou 2017).

In Lotus there are specific isoforms of key genes in the general phenylpropanoid (e.g. PAL or C4H) and flavonoid pathways (e.g. CHS, CHI) that are specifically involved in the metabolic channeling of carbon towards the flavonoid, isoflavonoid or lignin branch pathways. In S50 control plants increased light intensity has previously been found to reduce both PAL and CHS transcripts but to have no effect on DFR or ANS transcript levels, and, at least for the isoforms studied, transcript levels of PAL and CHS were not correlated with condensed tannin accumulation (Paolocci et al. 2005). In the work reported here, increased light intensity however resulted in a rapid and more than six-fold induction of a specific non-legume type chalcone isomerase transcript (*chi*2) in leaves of the low tannin S50 genotype, as determined

by qualitative RT-PCR, while the legume type *chi* genes (*chi1* and *chi3*) exhibited similar expression ratios before and after the increase in light intensity. In contrast, glutathione elicitation of *L. corniculatus* leaves which resulted in the accumulation of isoflavan phytoalexins, showed significantly increased expression of the two legume *chi* genes, *chi1* and chi3 while expression of *chi2*, the non-legume type chi did not vary (Lanot 2004**).**

**Lignin and lignin solubility**

Previous studies have shown that the reduced nutritional quality of some forage legumes is not solely due to condensed tannins, and that the adverse effects of condensed tannins may be combined with the detrimental dietary effects of lignin and that both factors should be considered when determining nutritive value (Barry and Duncan 1984; Miller and Ehlke 1996). For example, Barry and Manley (1986). established that in *L. pedunculatus* and *L. corniculatus* the reduced nutritive value under stress conditions was due to the simultaneous increase in the concentrations of lignin and condensed tannins, both of which depressed rumen carbohydrate fermentation and voluntary intake in sheep

The lower levels of TGA lignin in leaves, stems and roots of the high tannin genotype S41 grown at 10$^{o}$C compared with plants grown at 25$^{o}$C may suggests a decrease in total lignin in these tissues, or an apparent reduction in lignin due to the increased accumulation of starch and other non-structural carbohydrates. However, lignin may also be less soluble in TGA by growth at low temperatures, as thioglycolic acid derivatization involves the formation of thioethers of benzyl alcohol groups found in lignin, which enables it to be extracted from cell walls by alkali, and low temperature growth may change lignin structure resulting in lower TGA solubility.

**Carbohydrate accumulation**

The major effect of plant growth at 10ºC compared with growth at 25ºC in terms of carbohydrate accumulation was the 4-fold higher levels of root starch and reducing sugars available for utilization in shoot regrowth following defoliation in plants grown at 10ºC

Growth temperature has previously been shown to affect carbohydrate root reserve cycles in *L. corniculatus,* with plants grown at 18ºC being able to restore their root starch reserves while plants grown at 32ºC failed to do so (Nelson & Smith 1968). Spring herbage yields have also been found to be positively correlated with the mean non-structural carbohydrate levels in the two preceding years, with a cumulative effect of carbohydrate levels on growth (Alison & Hoveland 1989). Rapid metabolism of root reserve carbohydrates, such as starch may be necessary both for normal regrowth following defoliation and for tolerance to environmental stress, with root starch concentrations declining in defoliated plants, while increasing in roots of undefoliated plants (Boyce et al. 1992; Li et al.1996). Starch accumulation and degradation patterns in Lotus were strongly correlated with root amylase activity (Li et al. 1996), with defoliation of shoots resulting in an increase in the activities of taproot endoamylases, associated with the degradation of root starch in *Medicago sativa* and *L. corniculatus* (Volenec et al. 1991; Boyce et al., 1992).

It is evident that modulation of carbon partitioning in roots, leaves and stems between growth, storage carbohydrates and tannins is affected by temperature, and light intensity. Thus, changes in these environmental conditions which give rise to increased tannin or to decreased lignin solubility, may result in decreased forage digestibility. It has been suggested (Alison & Hoveland 1989), that management practises and breeding strategies should be designed to maintain high levels of root non-structural carbohydrates.

In subsequent experiments with the plants used in this study, we detected a major anomaly which suggested that the temperature at which the plants were grown was a major determinant of the ability of leaves and stems to accumulate phenolics in regrowth tissues following defoliation. The effect of different levels of root carbohydrate reserves on plant regrowth

following defoliation, and the effects of drought conditions and increased CO2 concentrations on phenolic accumulation and leaf and stem digestibility are reported in Part 4 of this series, elsewhere in this volume.

**Nutritional effects**

Condensed tannins are considered anti-nutritional at high concentrations (above 4-5% dry weight) because they reduce both digestibility and palatability (Barry and Duncan, 1984). In contrast, at low concentrations (2-3% dry weight) some condensed tannins are considered beneficial to ruminants as they bind to plant proteins and protect them from deamination by microbes present in the rumen, which reduces the possibility of bloat and increases the amount of protein available for post-ruminal digestion and absorption (Barry and Manley 1986; Waghorn et al, 1987).

Maximum tannin levels in leaves reached over 8% dry weight and in stems over 3% dry weight in the high tannin S41 genotype grown at 10oC, whereas maximum endpoint digestibility as determined by IVDMD and initial rates of digestion were found in plants grown at 25$^{o}$C,

The correlation between leaf tannin concentration and digestibility (IVDMD or initial rates), were not equally strong across all three genotypes (Table S1). This reduces the correlation coefficient for the mean data from all three genotypes. However, in the high tannin genotype S41 where tannin levels were consistently above 2.5% dry weight. correlation analysis showed a high negative correlation between initial rates of gas evolution ($R^2$ = -0.985), and total tannin concentration, but a lower correlation between IVDMD and total tannin concentration of leaves ($R^2$ = -0.452), and almost no correlation in stems ($R^2$ = -0.27 and -0.07 respectively) (Table S1). We have shown previously that correlations between the condensed tannin concentration of *L. corniculatus* leaves and the initial rate of gas evolution, were greater than with the pepsin-cellulase end-point digestibility method of determining IVDMD (Carter et al 1999), and that these correlations were only statistically significant in tissues with tannin levels above 2.5%,

which is entirely consistent with the finding that 2-3% dry weight of *L. corniculatus* tannin does not depress digestion and is beneficial as it increases the amount of protein available for post-ruminal digestion and absorption (Barry and Manley 1984; Waghorn et al. 1987).

Not all condensed tannins however have the same nutritional effect on ruminants. *L. corniculatus* tannins which consist of epicatechin (procyanidin) (67%) and epigallocatechin (prodelphinidin) (30%) extender units (Foo et al. 1996) do not appear to inhibit amino acid absorption (Wang et al. 1994) while those from *L. pedunculatus* with epigallocatchin (about 70%) as the dominant extender units (Foo et al. 1997) show extensive inhibition of uptake of most amino acids (Waghorn et al. 1987). Environmentally induced changes in tannin hydroxylation may therefore have significant effects on nutrient availability.

Both growth temperature and light intensity were found to modify the hydroxylation of tannins in *L. corniculatus*, and this may help to explain the high diversity of reports of the levels of tannin hydroxylation in this species.

Although the mechanisms regulating tannin hydroxylation are not completely understood (Dixon et al. 2005; He et al. 2008; Zhao et al. 2010), it is evident that tannin hydroxylation is environmentally controlled as both low temperature and high light intensity increase tannin hydroxylation. There is some evidence however that this may simply be due to changes in tannin biosynthesis in different cell types. Condensed tannin deposition in Lotus is restricted to specific cell types in leaves (Morris and Robbins 1992; Abeynayake et al. 2011), stems and roots (Morris and Robbins 1992) during their differentiation.

As condensed tannins are isolated from bulk plant tissues the original cellular location of specific tannin structures is unknown and their gross molecular structure (particularly the ratio of proanthocyanidin monomers which make up the tannin polymers), may therefore be derived from tannins with different structures from different cell types. The extent of the measured tannin hydroxylation may therefore be at least partially governed by the frequency of tannins

of different structures in different cell types in the tissue rather than in differences in tannin structure within individual cells.

**Tannin cellular deposition**

Under normal conditions, high tannin Lotus leaves can contain tannin cells in three positional locations, adjacent to vascular tissue, distributed through the palisade mesophyll and in a matrix formation in the spongy mesophyll (Robbins et al 2002) (see Fig S6). Over expression of the maize *Sn* transcription factor in the low tannin S50 genotype, which normally accumulates tannins in cells near the vascular tissue, resulted in increased tannin levels and induced the production of tannins in the palisade and spongy mesophyll cells (Robbins et al. 2002; Paolocci et al. 2005; Paolocci et al. 2007). This heterogeneous distribution of tannin cells in leaves may possibly be generated by local gradients of plant growth regulators as auxins have been found to suppress tannin accumulation in both shoot and root organ cultures of *L. corniculatus* at concentrations in the nmolar range (Morris and Robbins 1992),

The increase in condensed tannins at low temperatures or at high light intensity coupled with the evidence that higher tannin levels are a result of tannin accumulation in different cell types, and a high positive correlation ($R^2$ = 0.725) between the amount of tannin and the degree of tannin hydroxylation, indicates that it is highly likely that the different mesophyll cell types accumulate tannins with different molecular structures and with different degrees of hydroxylation.

This hypothesis is further supported by the observation that tissues containing tannins in only one cell type such as in seed coats or petals, have only one level of hydroxylation; either procyanindin in *Arabidopsis thaliana* seed coats (Debeaujon et al. 2003; Marinova et al, 2007) or in *Trifolium pratense*, and *Medicago truncatula* petals (Meagher, et al, 2006; Pang et al. 2007), as well as in *Lotus corniculatus* and *Lotus japonicus* petals (Morris, unpublished

observations), or prodelphinidin in *Trifolium repens*, *Trifolum fragiferum*, and *Trifolium ambiguum* petals (Foo et al, 2000; Meagher, et al, 2006).

## Conclusions

The differences in condensed tannin concentrations and tannin monomer hydroxylation between the three genotypes used in this study highlight the diversity found in Lotus species in generally and that this diversity and variability may be further increased by fluctuations in growth temperature and light intensity with subsequent impacts on nutritional quality. However, our results show that alone, currently predicted levels of summer temperature increase as a result of climate change, will probably not have a direct significant deleterious impact on the quality of *Lotus corniculatus* as forage, although this may be adversely affected if combined with other changing environmental factors such as increased $CO_2$ levels or by drought conditions, or as a result in warmer winters.

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## Author contributions

PM devised the project, carried out the tannin hydroxylation and wrote the manuscript. EBC produced the experimental material and carried out tannin and digestibility analysis. BH carried out HPLC for flavonoid quantification. AL carried out the light intensity work and transcript analysis and GA contributed to tannin and lignin analysis and edited the manuscript.

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## Figure Legends

**Fig. 1** Schematic of the flavonoid and lignin pathways showing the enzymatic steps leading to the major classes of end products, flavonoids, isoflavonoids, phenolic acids, phlobaphenes, anthocyanins, lignin and condensed tannins (proanthocyanidins).

**Fig. 2** Mean growth rates (g fresh weight $d^{-1}$ and cm $d^{-1}$), and maximum levels of tannin accumulation in leaves (**a**) and stems (**b**), and dry weight leaf: stem ratio (**c**) of the high, intermediate and low tannin genotypes (S33 S50 and S41) grown at 10ºC to 30ºC. Established clonal plants were defoliated to 5 cm and regrown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 4 -12 weeks. Mean values ± SE ($n$=9) (3x3 genotypes).

**Fig. 3** Effect of growth temperature on soluble and bound condensed tannin concentration of leaves (**a,b**) and stems (**d,e**) of the high S41 (**a,d**) and the low S50 (**b,e**) tannin genotypes and tannin solubility in leaves (**c**) and stems (**f**). Plants were grown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 4 -12 weeks. Different letters in the graph represents significant differences in total tannins between treatments ($P < 0.05$; $n = 3$).

**Fig. 4** Effect of growth temperature on tannin hydroxylation in leaves of high (S41) (**a**) and low (S50) (**b**) tannin genotypes and on the mean procyanidin: prodelphinidin (PC: PD) ratio (PP= propelargoniodin). Established clonal plants were defoliated to 5 cm and regrown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 4 -12 weeks.

**Fig. 5** Levels of starch (**a**) sucrose (**b**) and glucose + fructose (**c**) in leaves, stems and roots of the high tannin genotype (S41) grown at 10ºC or 25ºC. Established clonal plants were

defoliated to 5 cm and regrown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 6 weeks. Mean values ± SE ($P < 0.05$; $n$=3). * indicates significant difference from 10°C grown plants.

**Fig. 6** Effect of growth temperature on maximum levels of flavonol and flavonol glycoside accumulation by leaves of high (S41) (**a**) and low (S50) (**b**) tannin genotypes. Established clonal plants were defoliated to 5 cm and grown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 4 -12 weeks. Flavonoid glycosides were quantified as kaempherol-3-O-glucoside equivalents and aglycones as kaempherol. K-3-G = kaempferol-3-glucoside, K-3-R = kaempferol-3-rhamnoside, K-3-R-7-G = kaempferol-3-rhamnoside-7-glucoside, K-3,7-dR = kaempferol-3,7-dirhamnoside, K-7-R = kaempferol-7-rhamnoside, identified by reference to authentic standards.

**Fig 7** Effect of growth temperature on levels of thioglycolic acid soluble lignin, in leaves, stems and roots of the high tannin genotype S41. Established clonal plants were cut back and grown at 450 µmol $m^{-2}$ $s^{-1}$ with 18h day for 6 weeks. Different letters represent significant differences between treatments. Mean values ± SE ($P < 0.05$; $n$ = 3).

**Fig 8** Relationship between mean levels of tannin accumulation (**a**) and tissue digestibility as determined by IVDMD and initial rates of gas evolution (**b,c**) in a simulated rumen environment in leaves (**a,b**) and stems (**a,c**) of the high, intermediate and low tannin genotypes (S33 S50 and S41) grown at different temperatures. Mean values ± SE ($n$= 9) (3x3 genotypes)

**Fig 9** Effect of light intensity on accumulation of condensed tannins and hydroxycinnamic acid (HCA) esters (**a**) and tannin hydroxylation (**b**) in leaves of the low tannin genotype S50. Established clonal plants were defoliated to 5 cm and regrown at 20°C 16h day for 4 weeks at different light intensities. One stem from each of three plants was harvested and leaves assayed

for condensed tannins and HCA esters. PC-procyanidin; PD-prodelphinidin; PP-propelargoniodin. Total HCA esters were calculated as p-coumaric acid equivalents as acid hydrolysis gave p-coumaric acid as the only product. Mean values ± SE (*n*=3).

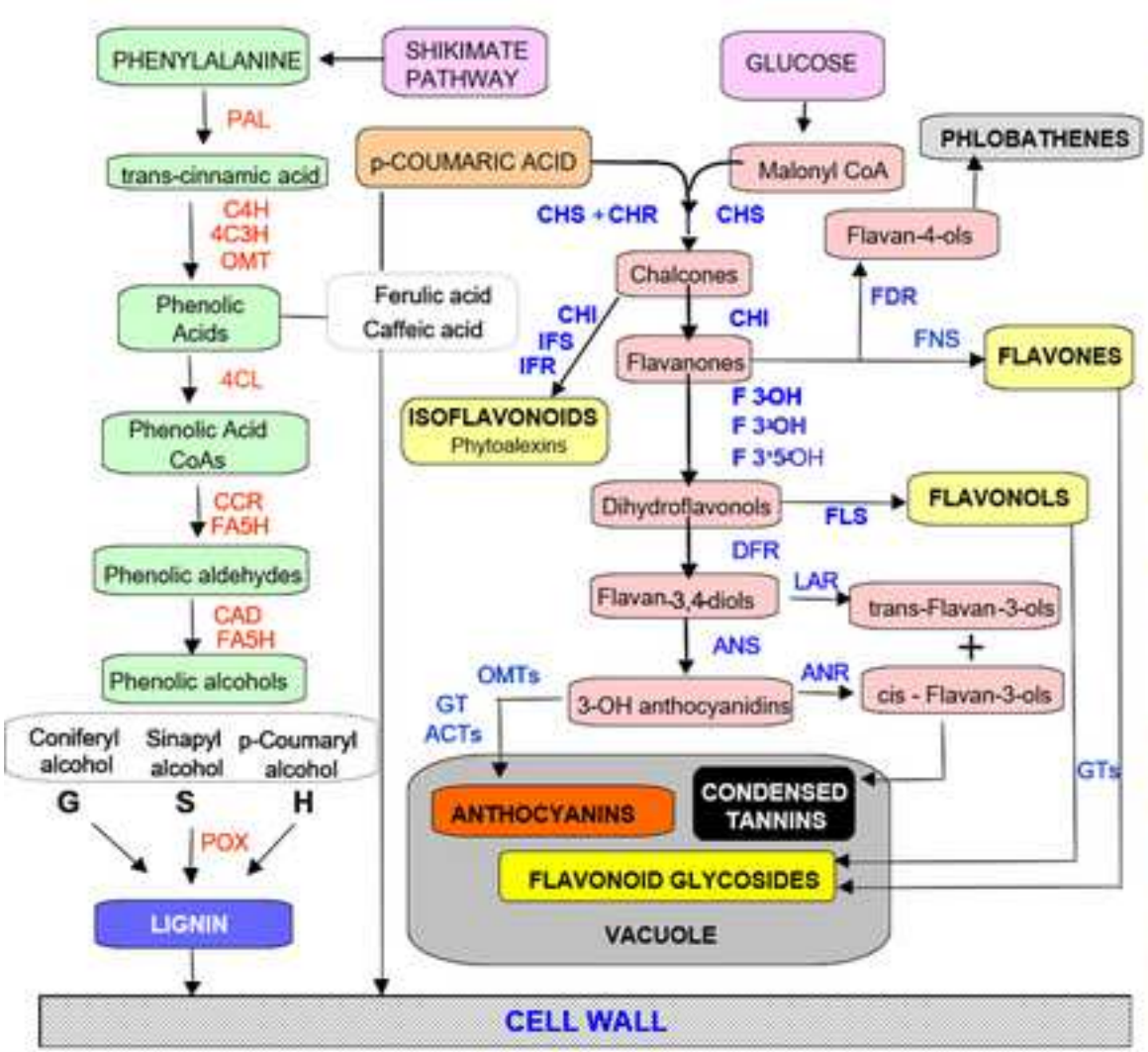
**Fig 10** HPLC and UV-vis spectrum of flavonols (1-9) and HCA esters (Y1-Y6) accumulated by leaves of the low tannin genotype S50 grown at 20 µm $m^{-2}$ $s^{-1}$ (**a**) or at 200 µm $m^{-2}$ $s^{-1}$ (**b**) light intensity. Analysis of UV/Visible spectra and HPLC profiles after acid hydrolysis showed that the peaks 1-9 were all kaempferol glycosides. Peak 1 = unknown kaempferol glycoside 4; Peak 2 = kaempferol-3-glucoside*; Peak 3 = kaempferol-3-rhamnoside*; Peak 4 = Peak 5 = unknown kaempferol glycoside 1; Peak 6 = kaempferol-3,7-dirhamnoside*; Peak 7 = unknown kaempferol glycoside 2; Peak 8 = unknown kaempferol glycoside 3; Peak 9 = kaempferol-7-rhamnoside*. * Peaks identified by reference to authentic standards. HCA esters (Y1-6) were identified as esters of p-coumaric acid as acid hydrolysis gave p-coumaric acid as the only product

**Fig 11** The effect of light intensities on flavonoid accumulation (**a**), the time course of flavonoid accumulation (**b**) and the expression ratio of three chi genes CHI1, CHI2, CHI3 normalised to β-actin (**c**), induced by high light intensity in leaves of the low tannin genotype S50. Established clonal plants were defoliated to 5cm stem height and regrown for 4 weeks at different light intensities (**a**) or regrown at 20$^{o}$C 16h day at 50 µmol $s^{-1}$ $m^{-2}$ for four weeks and then increased to 500 µmol $s^{-1}$ $m^{-2}$ (**b,c**). One stem from each plant was harvested and leaves assayed for flavonoids (*n*=3). Gene expression normalised to actin and relative to expression at t=0. K-3-G = kaempferol-3-glucoside*; K-3-R = kaempferol-3-rhamnoside*; K-3,7dR = kaempferol-3,7-dirhamnoside*; K-7-R = kaempferol-7-rhamnoside*,

* Peaks identified by reference to authentic standards. In this genotype acid hydrolysis gave kaempferol as the only product.

Figure 1






| | |
|---|---|
| CHS | chalcone synthase |
| CHI | chalcone isomerase |
| CHR | chalcone reductase |
| IFS | isoflavone synthase |
| IFR | isoflavone reductase |
| FDR | flavan 3,4-diol 4-reductase |
| FNS | flavone synthase |
| F 3-OH | flavanone 3-hydroxylase |
| F 3'-OH | flavonoid 3'-hydroxylase |
| F 3'5'-OH | flavonoid 3'5'-hydroxylase |
| FLS | flavonol synthase |
| DFR | dihydroflavonol –4-reductase |
| LAR | leucocyanidin reductase |
| ANS | anthocyanidin synthase (leucoanthocyanidin dioxygenase) |
| OMT | O-methyltransferase |
| GT | glycosyl transterase |
| ACTs- | acetyl transferases |
| ANR | anthocyanidin reductase; |
| PAL | phenylalanine ammonia-lyase |
| C4H | cinnamate-4-hydroxylase |
| 4C3H | 4cinnamate3-hydroxylase |
| OMT | O-methyltransferase |
| 4CL | 4-coumaroyl:CoA-ligase |
| CCR | Cinnamoyl-CoA reductase |
| FA5H | Ferulic acid 5-hydroxylase |
| CAD | cinnamyl alcohol dehydrogenase |
| POX | polyphenol oxidase |

Figure 2

Click here to access/download;Figure;Fig 2 PLAA-D-20-00713 Morris et al.tif

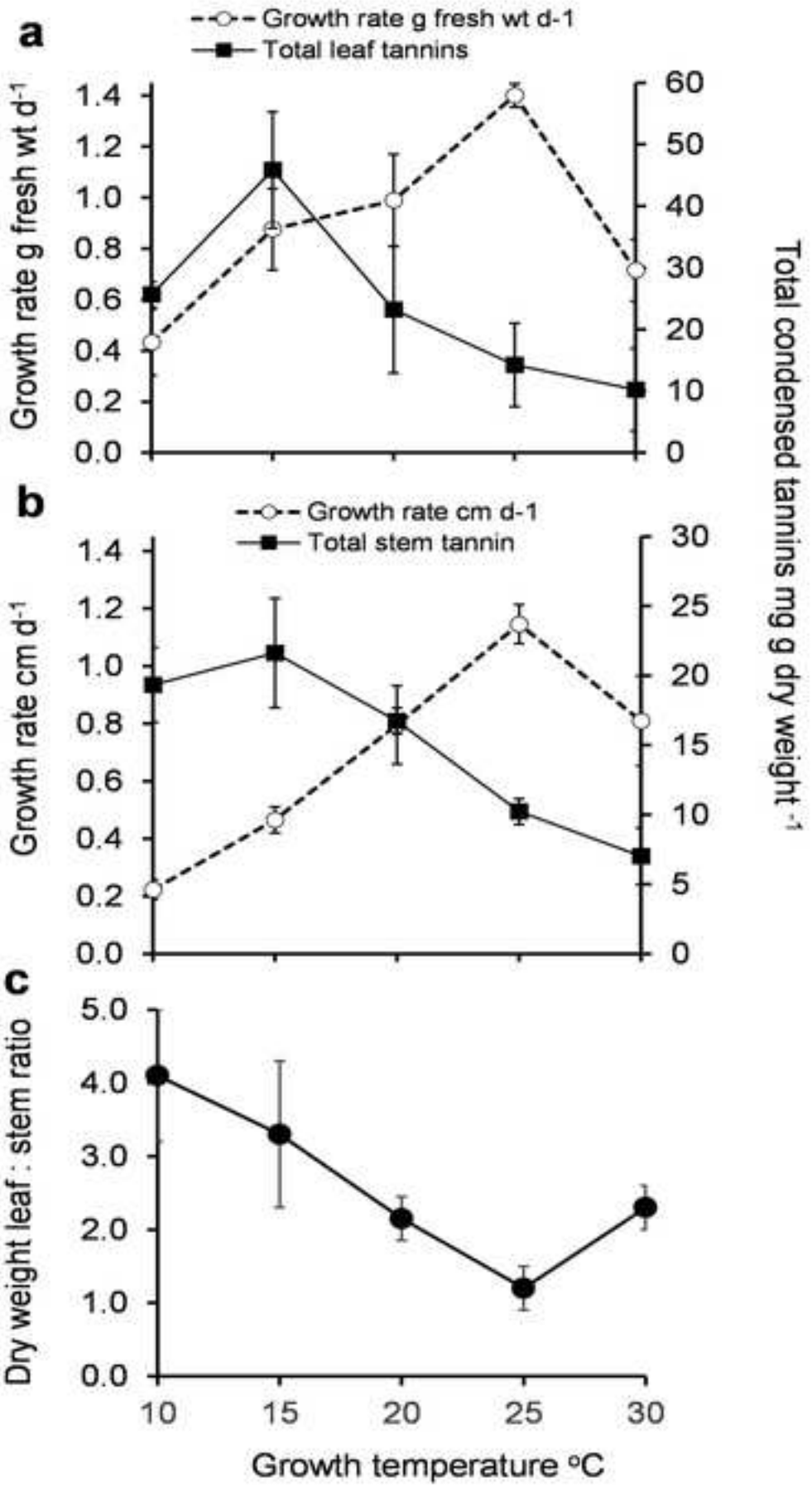


Figure 3



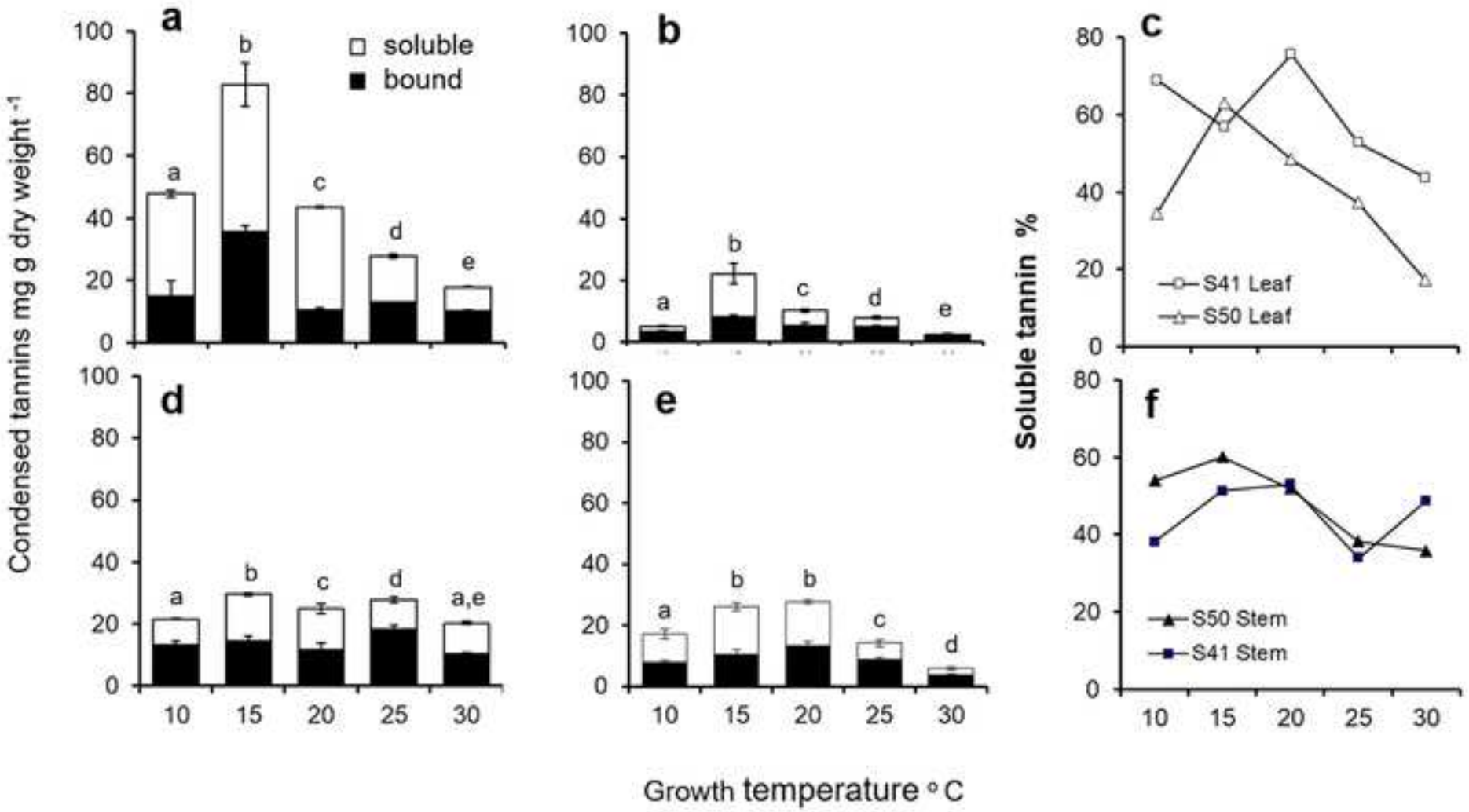


Figure 4



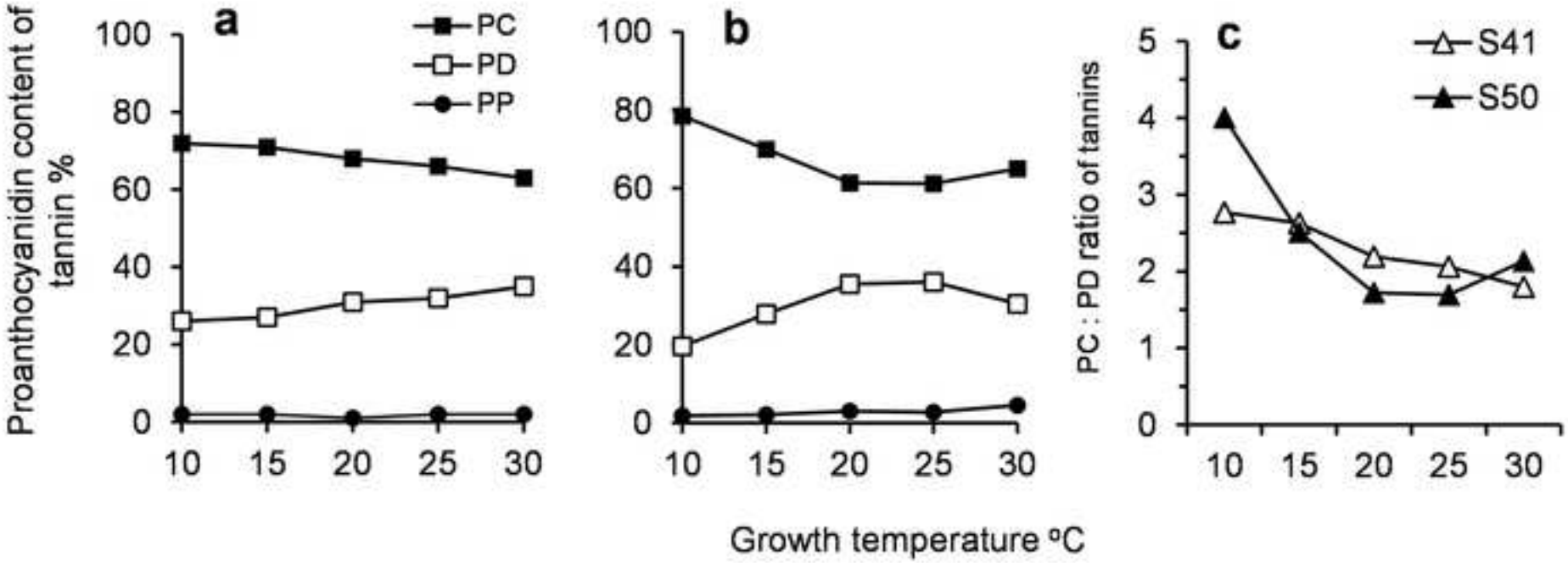


Figure 6

Click here to access/download;Figure;Fig 6 PLAA-D-20-00713 Morris et al.tif

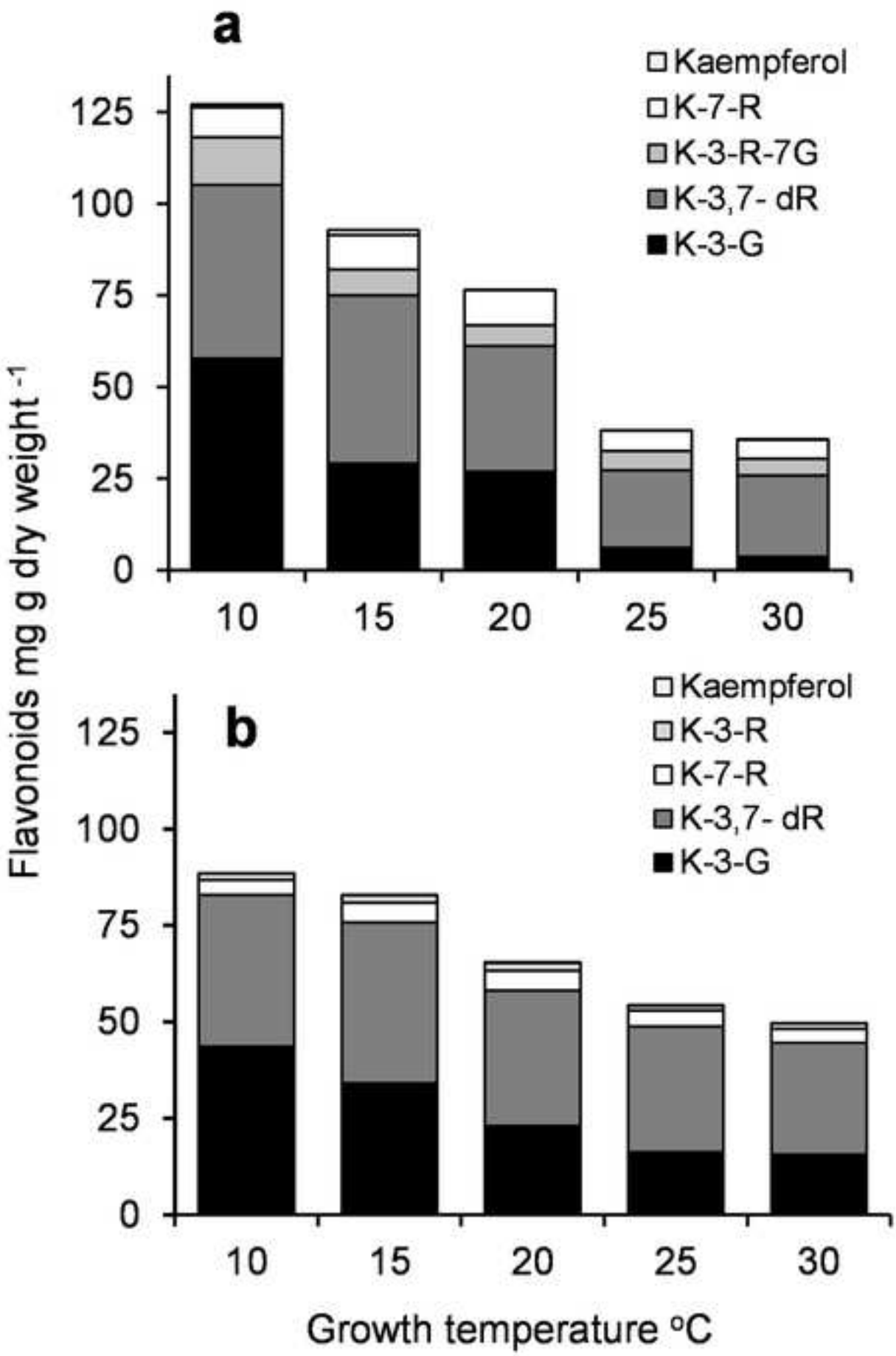


Figure 7



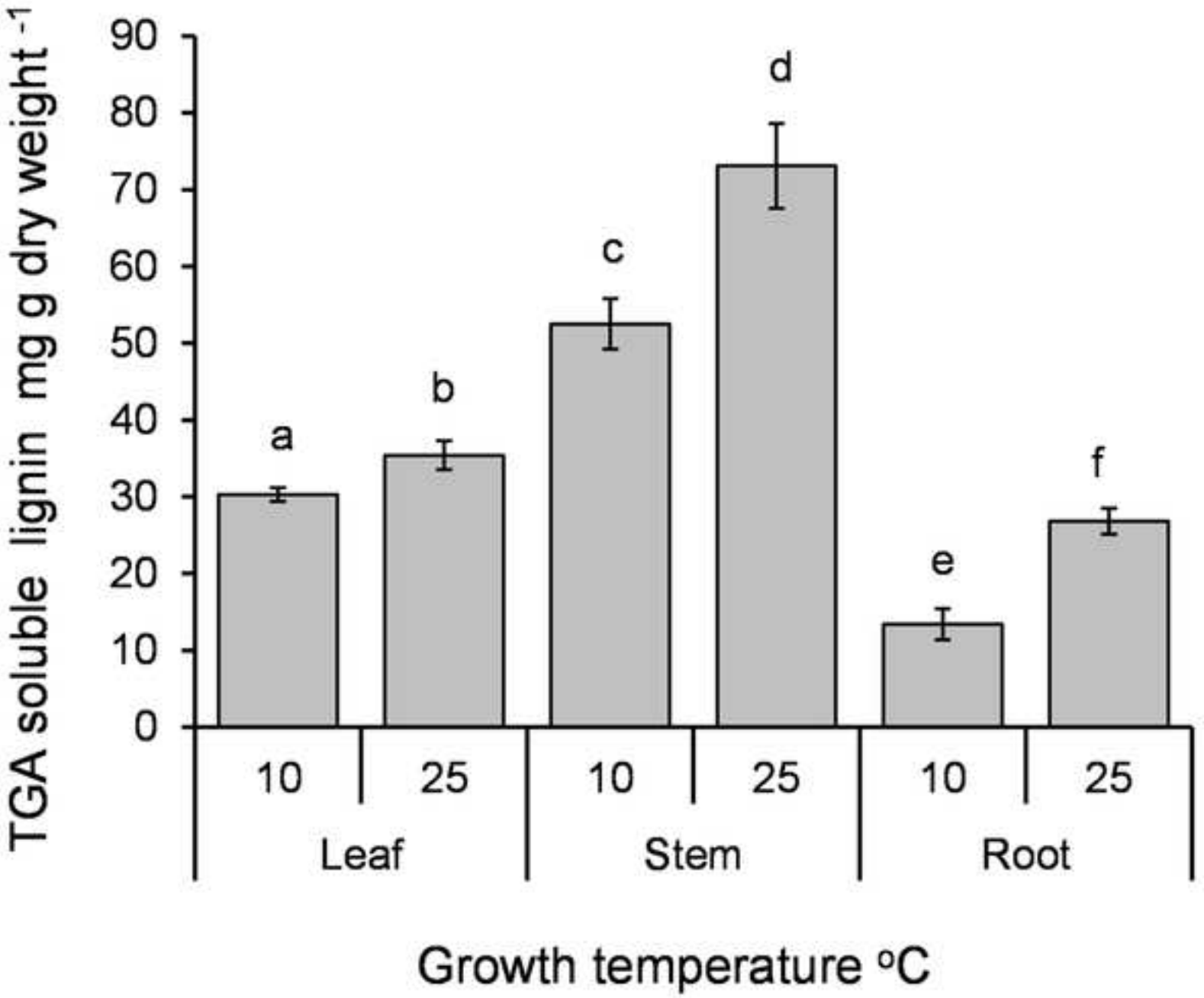


Figure 8

Click here to access/download;Figure;Fig 8 PLAA-D-20-00713 Morris et al.tif

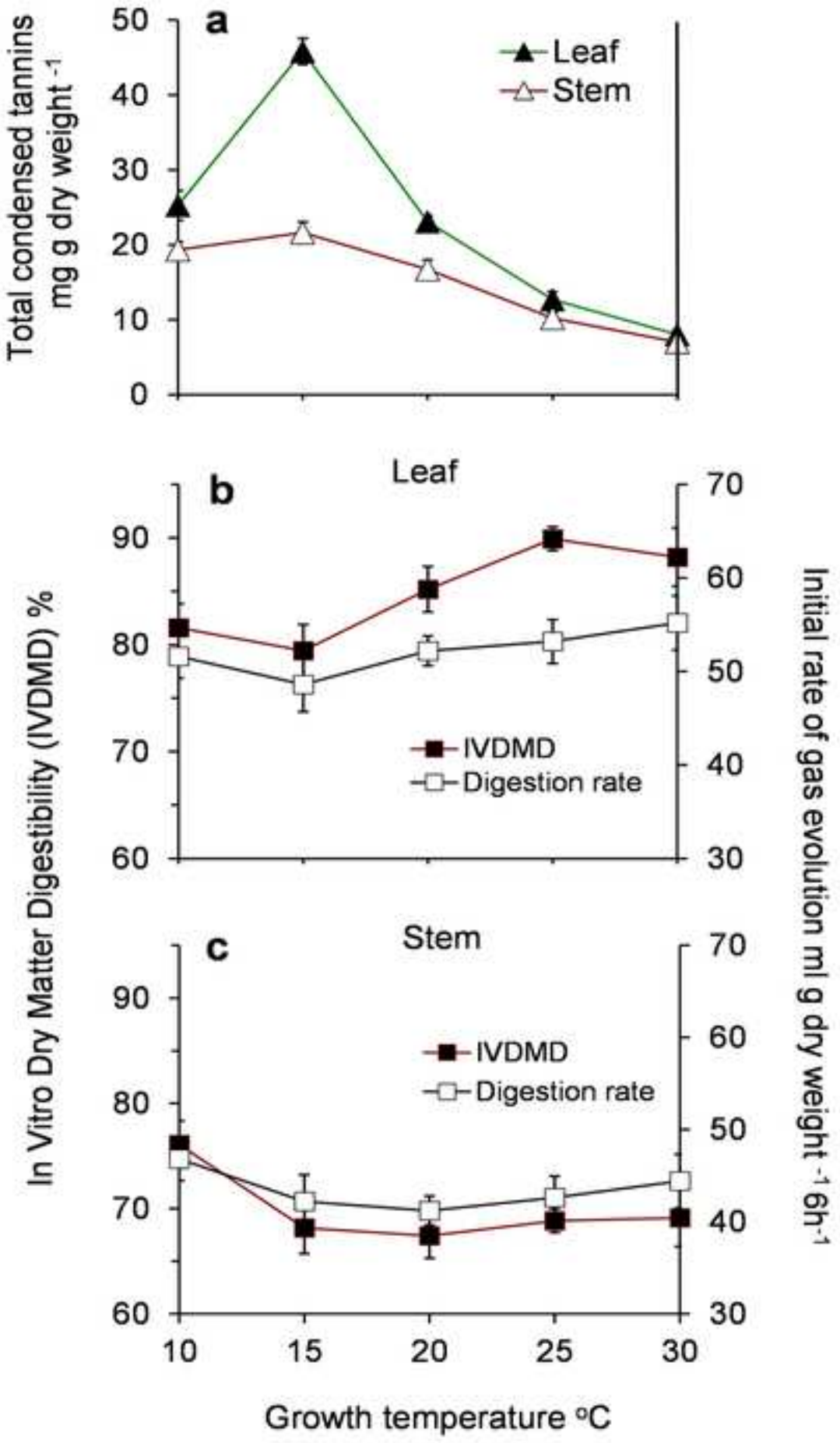


Figure 5



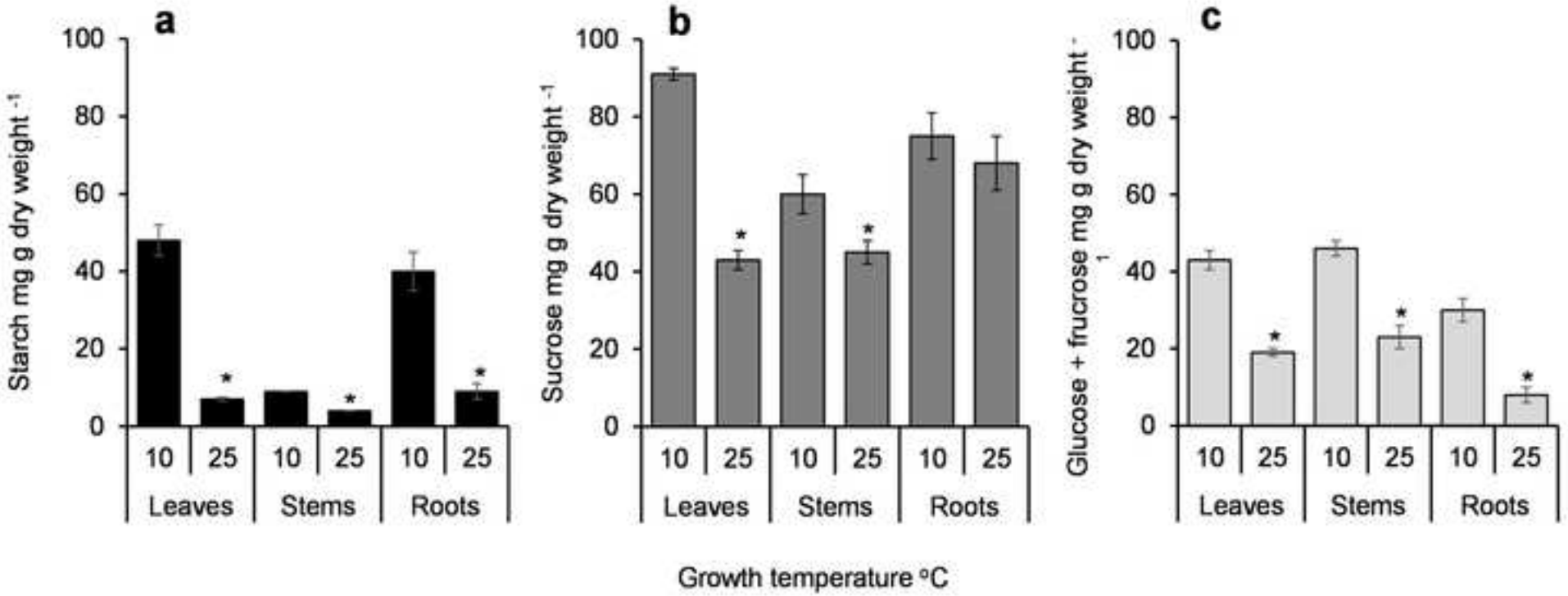


Figure 9



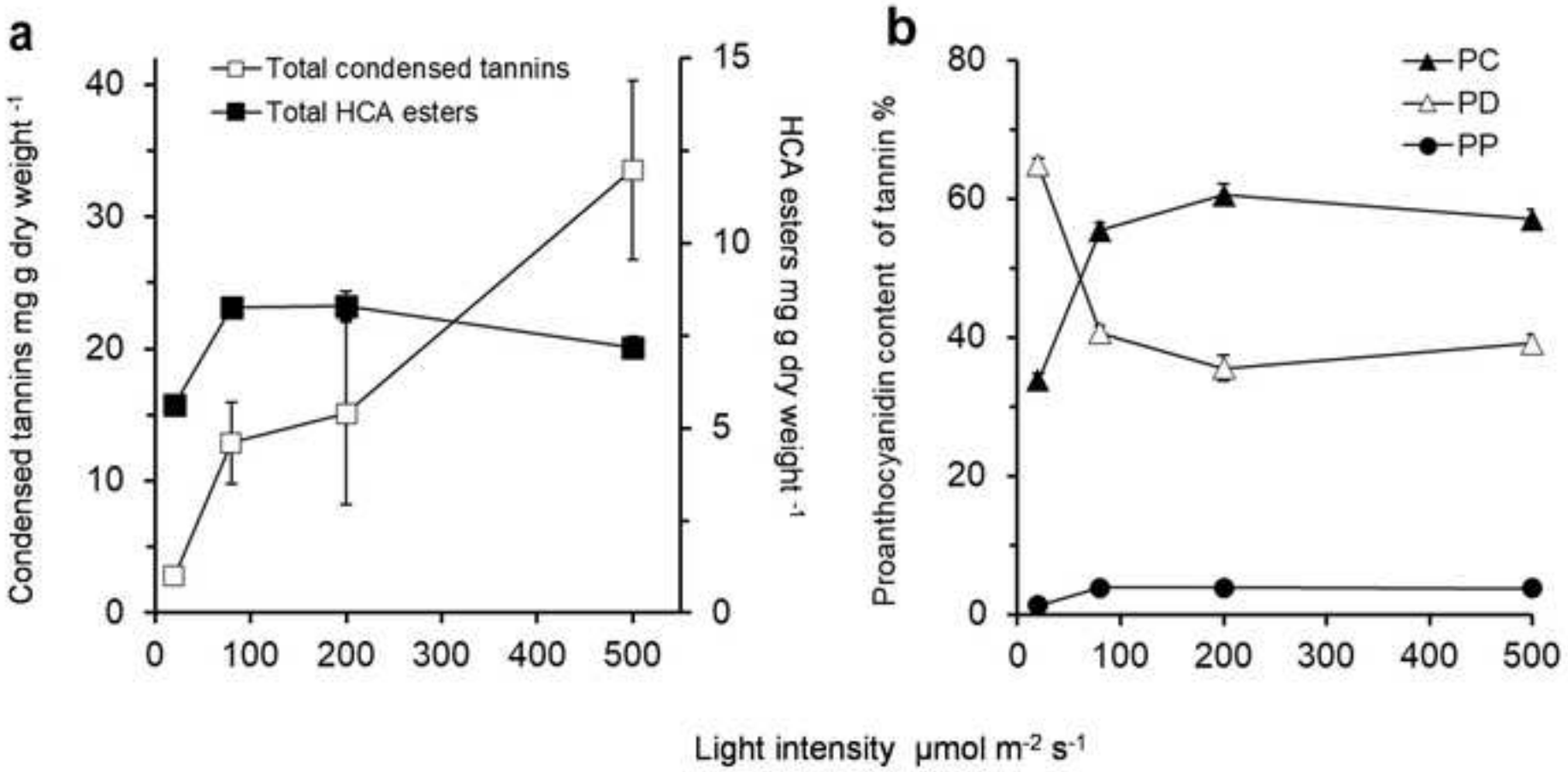


Figure 10



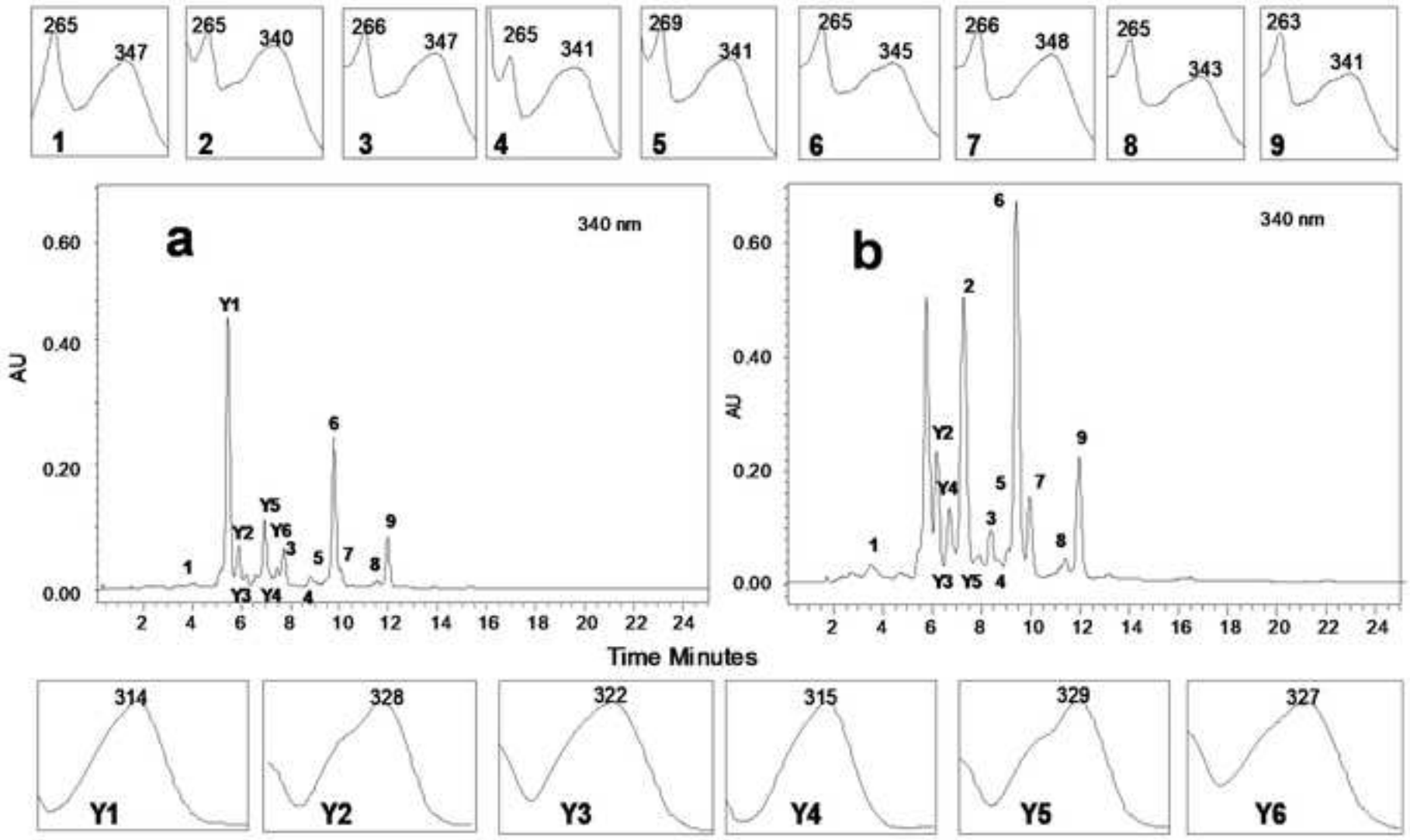


Figure 11