Rutin and quercetin content in the forage of common buckwheat as affected by maturity and conservation method

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Abstract

The content of rutin and quercetin was examined in fresh, hayed and ensiled forage of common buckwheat. The treatments were two varieties of buckwheat, Bamby and Lileja, and two ripening stages of harvest, first green and first brown achenes. In the silage, produced with experimental mini-silos, additional treatments were the wilting of the forage (at 35% dry matter) and the addition of *Lactobacillus plantarum* as inoculum. The concentration of rutin and quercetin decreased between ripening stages. Flavonoid content was different among varieties, Lileja had the highest rutin concentration (about 20 g kg^{-1}), while Bamby had the highest quercetin concentration (about 0.2 g kg^{-1}). Rutin and quercetin yield reached about 70 kg ha^{-1} and 500 g ha^{-1}, respectively. The haymaking process reduced the rutin and quercetin concentration in the forage, however the extent of reduction was higher at the green (-43% for rutin and -55% for quercetin) than at the brown achenes stage (-13% rutin and -26% quercetin). The ensiling process, with the associated bacterial activities, led to the transformation of rutin into quercetin. The decrease of rutin in the silage, compared to fresh forage, ranged between -84 and -99%, while in contrast the quercetin concentration increased by about 140-200 times. However, the loss of total rutin plus quercetin during ensiling was limited (approximately 5%). Forage wilting negatively affected rutin transformation in quercetin, while bacterial inoculum improved it. These results highlight that the forage of buckwheat could be considered like a dietary supplement rich of flavonoids, with the potential to be used as functional feed.

Keywords

Fagopyrum esculentum; flavonoids; hay; nutraceutical feed; silage.

Introduction

Common buckwheat (*Fagopyrum esculentum* Moench) is an annual dicotyledon herb belonging to the family Polygonaceae.

Buckwheat is mainly cultivated in the central and northern regions of Eastern Europe, China and the United States. In Italy buckwheat is cultivated in limited areas of the Alps and the Apennines, and its production is mainly for local consumption (Brunori *et al*. 2006; Tallarico *et al.* 2008).

There is renewed interest in buckwheat cultivation driven by the rising demand for its products, primarily gluten-free and high biological value flour (Kaur *et al*. 2015). The plant also contains flavonoids which can be used for the production of nutraceutical preparations and functional foods (Ahmed *et al*. 2014).

The main buckwheat flavonoid is rutin (quercetin-3-rutinoside) which has antioxidant, anti-inflammatory and anti-carcinogenic properties. In humans these properties can help reduce the fragility of blood vessels related to hemorrhagic diseases and hypertension (Oomah and Mazza 1996; Baumgertel *et al*. 2003).

Buckwheat as a whole plant can be used in ruminant feeding as a diet component for dairy cows (Amelchanka *et al*. 2010; Kälber *et al*. 2011; Mariotti *et al*. 2015), despite various irritating skin disorders described in light-colored animals when continuously exposed to sunlight (De Jong 1972). In livestock, rutin and quercetin can have positive effects, although a little different: the addition of rutin to diets of dairy cows tends to increase the milk yield and improve the digestibility of feed (Cui et al. 2015), while quercetin inhibits the growth of parasites and bacteria (Vijaya and Ananthan, 1996; Dupuy *et al*. 2003). Moreover the antibacterial activity of quercetin is enhanced by the presence of rutin (Arima *et al.* 2002). From the nutraceutical point of view, De Feo *et al*. (2006) showed that rutin provided by feeding plants is partially excreted in the goats milk, therefore providing the opportunity for

utilizing buckwheat forage to transfer flavonoids into foods that do not contain them, via the agro-livestock production chain.

Rutin content in buckwheat varies among the different plant parts: in the achenes it ranges from 0.2 to 0.4 g kg^{-1} dry matter (DM) (Kalinová and Dadáková 2006), in the inflorescences from 50 to 80 g kg^{-1} DM, in the leaves from 30 to 70 g kg^{-1} DM, and in the stalks from 7 to 12 g kg^{-1} DM (Baumgertel *et al.* 2010; Kalinová and Dadáková 2013). The same pattern of distribution among the plant organs is true for quercetin, although with much lower values (Zielińska *et al*., 2012). Thus, the herb contains much more rutin and quercetin than the kernels.

The optimal time of buckwheat harvest in order to obtain the highest rutin and quercetin concentration and yield is not clear. Metzger *et al.* (2010) reported that the maximum rutin concentration is achieved in the flowering stage of buckwheat. The flowering stage, however, lasts for a long time, due to the indeterminate growth habit of this species. During this period, the plant composition and structure and the forage yield change markedly, which, in turn, could affect the flavonoid content of the forage (Goering *et al*. 1972; Mariotti *et al*. 2015).

There is little information on the influence of conservation methods, such as hay or silage, on rutin and quercetin concentrations in buckwheat. After plant harvest, the activity of endogenous enzymes, such as rutinosidase and polyphenol oxidases, might contribute to the degradation of rutin and quercetin (Baumgertel *et al.* 2010). In addition, treatments commonly used before ensiling forage, such as wilting or the use of starter cultures, could also affect rutin and quercetin concentrations in the ensiled buckwheat.

The aim of this study was to evaluate: i) the optimal phenological stage of harvest in order to maximize rutin and quercetin concentration and yield; ii) the influence of different conservation methods, hay and silage, on rutin and quercetin concentration; iii) the effect of

wilting and the addition of a bacterial starter on rutin and quercetin concentration in the silage. We tested the rutin and quercetin concentrations of two commercial varieties of common buckwheat used for fresh, hayed and ensiled forage production at different maturity stages.

Materials and methods

The trials were carried out in two consecutive years, 2013 and 2014, in open fields and in the laboratory. Open field trials were carried out at the Enrico Avanzi Interdepartmental Centre of Agro-Environmental Research (CIRAA) of the University of Pisa (43° 40' N, 10° 19' E). The main soil physical and chemical properties were 43.4 % sand, 38.8 % silt, 17.8 % clay (Gee and Bauder 1986), 7.5 pH (McLean 1982), 21.1 g kg^{-1} organic matter (Walkley and Black method, Nelson and Sommers 1982), 1.71 g kg^{-1} total nitrogen (Kjeldahl method, Bremner and Mulvaney 1982), 6.6 mg kg^{-1} available P (Olsen method, Olsen and Sommers 1982), 128.1 mg kg^{-1} available K (ammonium acetate test method, Thomas 1982).

In each year, treatments were as follows: two stages of maturity at harvest, stage 70 (first green achenes) and stage 85 (first brown achenes) of the Arduini *et al*. (2016) scale, and two common buckwheat varieties, Bamby and Lileja. These stages were chosen because they are near to and after full flowering, which is considered as the phase characterized by the maximum concentration of flavonoids in the plants (Cawoy *et al.* 2009), and because have the advantage of being easily detected. The experiment was set in a split-plot design with harvest stage as the main plot and variety as the sub-plot. The sub-plot dimension was 48 m^2 (6 x 8 m). Each treatment was replicated three times.

In both years buckwheat was sown in April, with a 14 cm row spacing at a density of 250 viable achenes per m^2. Nitrogen, phosphorous and potassium fertilizers were applied at

rates of 40, 44 and 83 kg ha^{-1}, as urea, triple superphosphate and K_2SO_4 respectively. Nitrogen was applied just before seeding, whereas P and K were applied before tillage. Harvest occurred at about 9 and 11 weeks after sowing, for green and brown achenes, respectively. Fresh weight yield was determined in a swath of 1 by 5 m, cut through the center of each sub-plot. One forage sample of 1 kg was collected, dried at 50 °C to constant weight, weighed to determine DM yield and analyzed to quantify rutin and quercetin concentration. Rutin and quercetin yield was calculated by multiplying rutin and quercetin concentration by DM yield.

The hay (85% DM, approximately) was prepared in the field following the conventional haymaking technique used in the area. A 1 kg sample of buckwheat hay from each sub-plot was collected, dried at 50 °C to constant weight, and used for rutin and quercetin determination.

The ensiling experiment was performed in a laboratory, with a 1 L anaerobic glass jar mini-silo. A portion of the fresh forage harvested from each sub-plot was immediately collected and another portion was thinly spread on black plastic sheets for 24 h in order to obtain unwilted and wilted (35% DM, approximately) forage, respectively, for use in the ensiling experiment. The inoculated silage, used for the uninoculated *vs* inoculated comparison, was prepared by adding an inoculum composed of four bacterial strains belonging to the *Lactobacillus plantarum* species (60A, 60H, 42A and A43 – *L. plantarum* collection of the Department of Veterinary Science, University of Pisa) to the wilted and unwilted forage, applied at a rate of 10^6 cfu g^{-1} of fresh matter. *L. plantarum* inoculum was prepared as follows: the strains were maintained at -80 °C on de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) with 15% glycerol and revitalized by two passages in MRS broth. An inoculum at 1% of each of the four strain cultures in the same broth was harvested after an overnight incubation at 37 °C in anaerobiosis, for the forage inoculation. All forages

were chopped into 2–3 cm pieces with a laboratory chopper, and ensiled at about 300 kg DM m^{-3} density. All silages were stored at 20 °C for five months. The final pH of all the silages was measured (aqueous silage extract). For rutin and quercetin quantification, samples were dried at 50 °C to constant weight.

The ensiling experiment was carried out by comparing two years (2013 and 2014), two stages (green and brown achenes), two varieties (Bamby and Lileja), two wilting conditions (unwilted and wilted) and two fermentations (uninoculated and inoculated with lactic acid bacteria starter). Each treatment was replicated three times, resulting in 48 silages per year.

On all forages, rutin and quercetin quantification was performed by high performance liquid chromatography (HPLC) analysis following Steadman *et al*. (2001) with some modifications. Extraction was carried out with 20 mL of 100% methanol on 250 mg and 100 mg of dried sample, for fresh and hay and for silage, respectively, after the addition of 100 µL of vanillin (300 mg mL^{-1} in methanol) used as an internal standard. After filtration with 0.20 µm filters, 20 µL of the extract were injected into the chromatographic system. HPLC analyses were carried out using a Jasco HPLC apparatus (Jasco Corporation, Japan) equipped with two gradient pumps (PU-1580), a mixer unit (HG-2080-03), and a UV detector (870-UV) set at 350 nm. The stationary phase was a RP Luna C18 column (250 mm × 4.60 mm × 5 µm) (Phenomenex, Torrance, CA, U.S.A.). The mobile phase contained acetonitrile/acetic acid (95:5 v/v) (A) and water (B) and was eluted at a flow rate of 0.5 mL min^{-1} using the following gradient: time = 0 min, 20% A; time = 24 min, 52% A; time = 30 min, 100 % A; time = 34 min, 100% A. Rutin trihydrate, quercetin and vanillin solutions (Sigma-Aldrich Inc., St. Louis, MO, USA) in methanol were used for chromatographic peaks identification and calibration curves calculations. The retention times of rutin, vanillin and quercetin were 14.0, 18.4 and 25.5 minutes, respectively. Figure S1 shows sample HPLC chromatograms obtained in the analysis of fresh, hayed and ensiled forage of buckwheat,

with several identified peaks.

Data were statistically analyzed by analysis of variance (ANOVA). For rutin and quercetin concentrations and yield of the fresh forage and hay, ANOVA was performed to test differences between years (2013 and 2014), stage of harvest (green and brown achenes), variety (Bamby and Lileja) and their interactions. For rutin and quercetin concentrations of the silages, ANOVA was performed to test differences between years, stage of harvest, variety, wilting (unwilted and wilted), fermentations (uninoculated and inoculated) and their interactions. The combined analysis over years was conducted after verifying the homogeneity of error variances by the chi-square test. All treatments were considered as fixed effects. Significantly different means were separated at the 0.05 probability level by the least significant difference test (Steel *et al.* 1997).

Results

There were no significant differences between years, or interactions between years and other treatments for any of the variables measured. Thus, the average values of the two years were reported.

Rutin and quercetin concentration in fresh and hayed forage

Rutin concentration in the DM of the fresh forage decreased by 11% from the stage of green achenes to brown achenes (Table 1), and was approximately 12% higher in Lileja than in Bamby (Table 2).

Rutin concentration in the DM of the hay was lower than in fresh forage. Rutin values also showed an increase of about 38% from the green achenes to the brown achenes stage (Table 1), and were 6% higher in Lileja than Bamby (Table 2).

Quercetin concentration in the fresh forage was notably lower than the rutin concentration, by about 150 times. Quercetin concentration decreased more markedly with the age of the plant compared to rutin, but in a different way in Bamby (-50%) than in Lileja (-30%) (Figure 1). In addition, the quercetin concentration, unlike rutin, was always higher in Bamby than in Lileja.

In the hay the quercetin concentration decreased with the age of the plant, although only slightly (Table 1). Bamby showed higher quercetin concentrations than Lileja (Table 2).

Both in fresh and hayed forage, rutin was the most abundant flavonoid, representing more than 99% of the sum of rutin and quercetin concentrations (RQ).

Rutin and quercetin yield

The maximum rutin yield obtained with the fresh forage of buckwheat was above 65 kg ha^{-1}, and was obtained with the Bamby variety harvested in the brown achenes stage. This value was 30% higher than that obtained in the green achenes stage, and 16% higher than that obtained with Lileja (Tables 1 and 2).

On the other hand, the maximum quercetin yield, corresponding to about 500 g ha^{-1} for Bamby and to 200 g ha^{-1} for Lileja, was obtained in the green achenes stage, after which values remain unchanged in Lileja and decreased in Bamby (Figure 1).

Rutin and quercetin concentration in ensiled forage

In buckwheat silage, pH values ranged from 3.82 to 4.20, with lower values recorded for unwilted forage, and higher values were recorded for the wilted forage harvested in the brown achenes stage (Figure 2). The addition of *L. plantarum* inoculum resulted in a significant decrease in pH values from 4.04 to 3.83, as a mean of years, stages, variety and

wilting treatments (data not shown).

Rutin concentration in buckwheat silage was markedly lower than that observed in fresh forage. In the unwilted silage, the rutin concentration did not change from the green to the brown achenes stage (about 0.2 g kg^{-1}), while in the wilted silage, values increased to 3 g kg^{-1} (Figure 3).

Unlike rutin, the quercetin concentration in the silage was much higher than in the fresh forage and in the hay. In the green achenes stage, quercetin reached concentrations slightly above 18 g kg^{-1} for both wilted and unwilted silage (Figure 3). Quercetin concentration decreased in the brown achenes stage, and more markedly in the wilted (-21 %) than in the unwilted silage (-17%). As a result, while in the green achenes stage there was no difference in quercetin concentration between wilting treatments, in the brown achenes stage, the quercetin concentration was 10% higher in the unwilted silage. The quercetin concentration in the silage, as a mean of all the other treatments, was higher for Lileja (17.6 g kg^{-1}) than for Bamby (16.6 g kg^{-1}).

Regarding the use of a bacterial starter culture, as a mean of all other treatments, the rutin concentration was significantly lower in the inoculated than in the uninoculated silage, while the quercetin concentration showed the opposite trend (Figure 4).

The RQ concentration decreased by about 14% from the green to the brown achenes stage, and was higher in Lileja than in Bamby (Tables 1 and 2).

In the silage, unlike the fresh forage and hay, quercetin was the most abundant flavonoid, representing more than 99% of RQ in the unwilted silage, and no less than 83% of RQ in the wilted silage. The quercetin in the silage, as a proportion of RQ, was highly correlated ($r = 0.83^{**}$) with the pH values (Figure 5).

Discussion

The concentration of rutin and quercetin in the forage of buckwheat was not different between the two years. This is probably because differences in rainfall and temperature between the two years were negligible: total rainfall from April to June was 151 mm in 2013 and 145 mm in 2014 and, in both years, the ten-day mean temperature varied from 13 to 23 °C, and the mean temperature of the entire cycle was 18 °C.

In the fresh forage of common buckwheat, the maximum concentrations of both rutin and quercetin (20 and 0.2 g kg^{-1} DM, respectively) were obtained at the green achenes stage, and decreased with the increasing age of the plants. Similar results were obtained for rutin in Germany by Baumgertel *et al*. (2010) and in Iraq by Ghouzhdi *et al*. (2009). Higher rutin values were obtained by Kalinová and Dadáková (2013) when delaying the sowing by a month, probably due to the higher solar radiation intensity. However, in Mediterranean plain areas and in rainfed conditions, delaying the sowing date can result in water stress which limits the growth of buckwheat. We found that the quercetin concentration was always very low, as already reported in other studies (Zielińska *et al*., 2012).

Bamby and Lileja varieties showed differences in flavonoids synthesis ability: the rutin concentration was higher in Lileja than in Bamby, while the reverse was true for quercetin. These results confirm those previously reported by Kitabayashi *et al*. (1995) and Kalinová and Dadáková (2006). On the other hand, quercetin concentration variability among varieties has not been clearly demonstrated in previous studies. Our results seem to suggest that the quercetin concentration was also variable between varieties, although with a different ranking than rutin.

In order to obtain the highest rutin and quercetin yield per hectare, corresponding to about 70 kg ha^{-1} and 0,3-0,5 kg ha^{-1}, it is preferable to harvest the plants at the brown achenes stage and use the Bamby variety, since these are the conditions which led to the

highest dry matter yield in our experiments and this research has clarified that rutin and quercetin yield were more affected by DM production, rather than by flavonoids concentration in plant tissues. The yield of flavonoids obtained in our study was higher than that observed in Poland by Golisz *et al.* (2007).

The haymaking process led to a reduction in rutin content in the forage. There was a higher decrease for forage harvested at the green achenes stage (-43%), than for the forage harvested at the brown achenes stage (-13%). This is likely due to mechanical losses, which normally occur during haymaking, which are higher the earlier the plant is harvested. These losses mainly affect the most delicate organs of the plant, such as leaves and flowers, which, however, are also those with the highest rutin concentration (Kalinová and Dadáková 2006; Zielińska *et al.* 2012). Likewise, Mariotti *et al*. (2015) observed that haymaking causes greater losses of crude protein and total digestible nutrients in the green achenes stage rather than in the brown achenes stage, presumably for the same reason.

The quercetin concentration was affected even more by haymaking, with losses ranging from 26% in the brown achenes stage to 55% in the green achenes stage.

Rutin was the most abundant flavonol both in fresh and hayed forage, representing about 99% of the total RQ content.

The silage of common buckwheat reached a satisfactory acidification, except for the wilted forage harvested at the brown achenes stage (pH > 4). Ensiling resulted in rutin degradation and in an increased quercetin content. In ensiled forage, the rutin concentration was very low compared to the fresh forage, with a decrease ranging from -84% to -99%. Conversely, the quercetin content increased 140-200 times. As a result, quercetin was the most abundant flavonol in the ensiled forage of buckwheat, with percentages ranging from 84% to 99%. Surprisingly, quercetin as a proportion of RQ, was quite negligible in the fresh forage but increased progressively in the silage up to 100% as the pH values decreased.

Thus, the rutin decreases and quercetin increases were probably due to a more abundant lactic acid bacteria proliferation. The use of a bacterial inoculum was in fact linked to a decreased pH, a lower rutin content and a higher quercetin content. Since rutin is the glycoside resulting from the bond between the flavonol quercetin and rutinose (a disaccharide), it is likely that lactic acid bacteria promoted the breakage of the glycosidic bond and the release of quercetin either directly with their metabolism or indirectly, as a result of the changes in the silage pH and composition. Other effects of the same bacterial inoculum in the silage of buckwheat were reported in another research and can be summarized with an increase of the lactic acid and a decrease of acetic acid and N-NH_3 (Mariotti et al., 2015).

The total RQ in ensiled forage, compared to fresh forage, decreased by about 5%, which is much lower than that observed for hayed forage. Compared with other studies, the flavonol losses were slightly higher than those observed for crude protein and lower than those reported for the nutritional value (Mariotti *et al*., 2015).

All summarized, this study highlight that the forage of buckwheat could be considered like a dietary supplement rich of rutin, in the form of fresh forage and hay, or quercetin in the form of silage; thus, It may have the potential to be used as nutraceutical feed, to inhibits the growth of parasites and bacteria in livestock, or to transfer rutin and/or quercetin in food products of animal origin.

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Tables

Table 1 Rutin concentration and yield, quercetin concentration and sum of rutin and quercetin concentration (RQ) in the dry mater (DM) of fresh, hayed and ensiled forage of buckwheat, as affected by stage of harvest. Data are means of two years, two varieties, and three replicates.

Stage	Fresh forage		Hay		Silage
	Rutin		Rutin	Quercetin	RQ
	(g kg^{-1} DM)	(kg ha^{-1})	(g kg^{-1} DM)	(g kg^{-1} DM)	(g kg^{-1} DM)
Green achenes	19.8 a	53.1 b	11.2 b	0.06 a	19.5 a
Brown achenes	17.7 b	69.0 a	15.4 a	0.05 b	16.7 b

Within a column, values followed by the same letter are not statistically different for $P < 0.05$.

Table 2 Rutin concentration and yield, quercetin concentration and sum of rutin and quercetin concentration (RQ) in the dry matter (DM) of fresh, hayed and ensiled forage of buckwheat, as affected by variety. Data are means of two years, two stages of harvest, and three replicates.

Variety	Fresh forage		Hay		Silage
	Rutin		Rutin	Quercetin	RQ
	(g kg^{-1} DM)	(kg ha^{-1})	(g kg^{-1} DM)	(g kg^{-1} DM)	(g kg^{-1} DM)
Bamby	17.7 b	65.7 a	12.9 b	0.07 a	17.8 b
Lileja	19.8 a	56.4 b	13.7 a	0.04 b	18.5 a

Within a column, values followed by the same letter are not statistically different for $P < 0.05$.

Figures

Figure 1 Quercetin concentration and yield in the dry matter of fresh forage of buckwheat, as affected by the interaction of harvest stage x variety. Data are means of two years, and three replicates. Vertical bars represent LSD at P < 0.05.

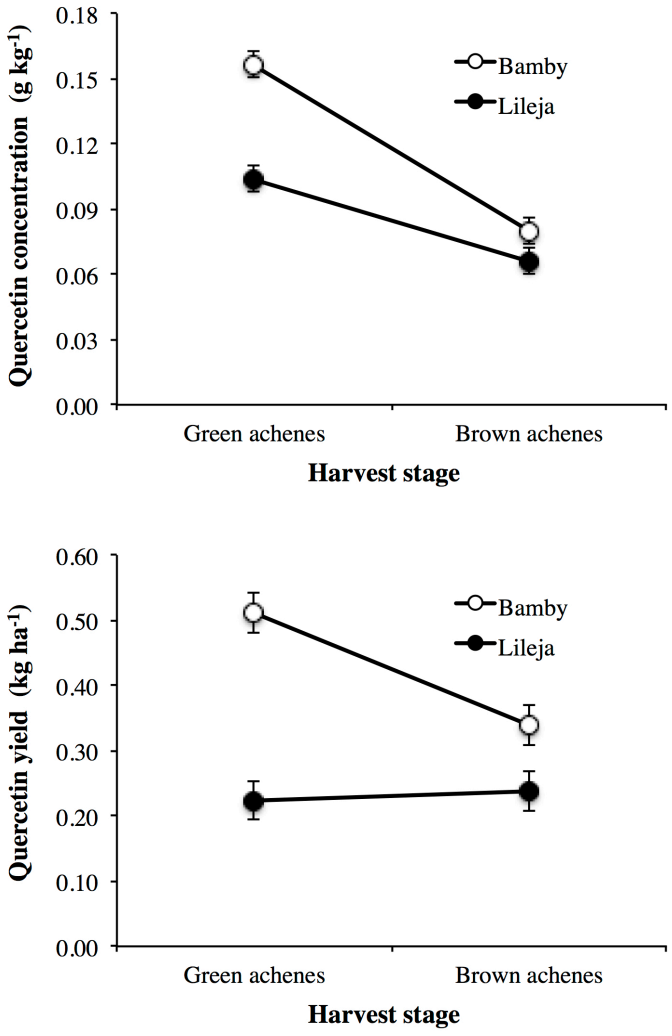


Figure 2 pH of silage of buckwheat, as affected by interaction of harvest stage x wilting. Data are means of two years, two fermentations, two varieties, and three replicates. Vertical bars represent LSD at $P < 0.05$.

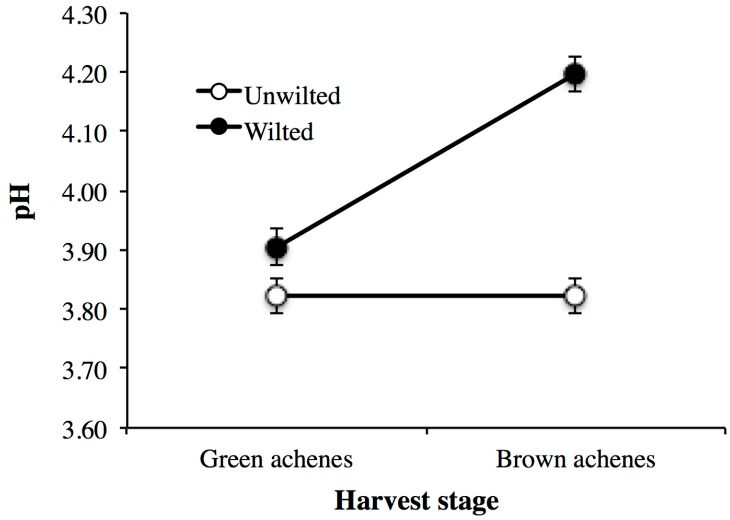


Figure 3 Rutin and quercetin concentration in silage of buckwheat, as affected by interaction of harvest stage x wilting. Data are means of two years, two fermentations, two varieties, and three replicates. Vertical bars represent LSD at P < 0.05.

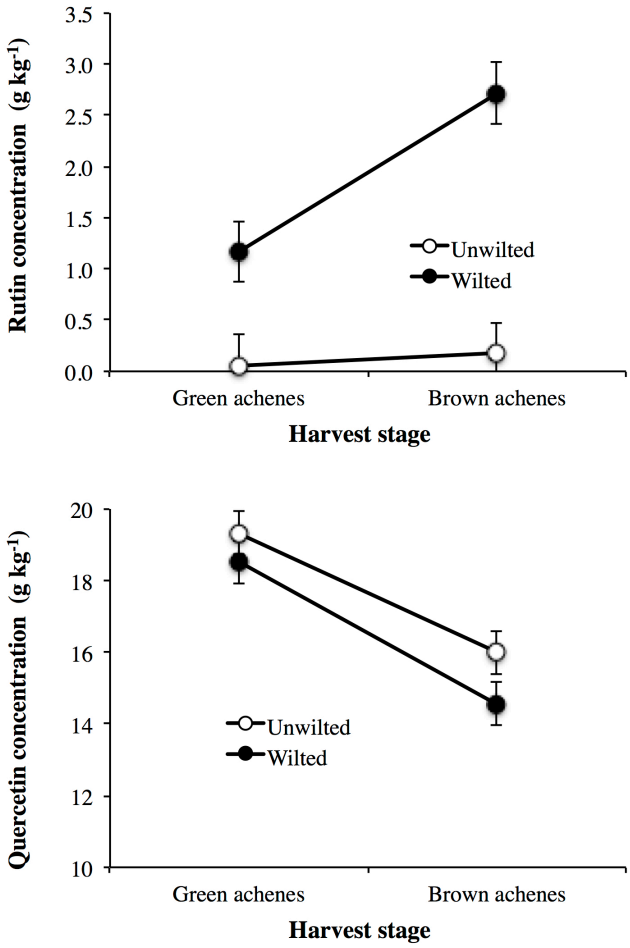


Figure 4 Rutin and quercetin concentration in the silage of buckwheat as affected by fermentations. Data are means of two years, two stages, two varieties, and three replicates. Vertical bars represent LSD at $P < 0.05$.

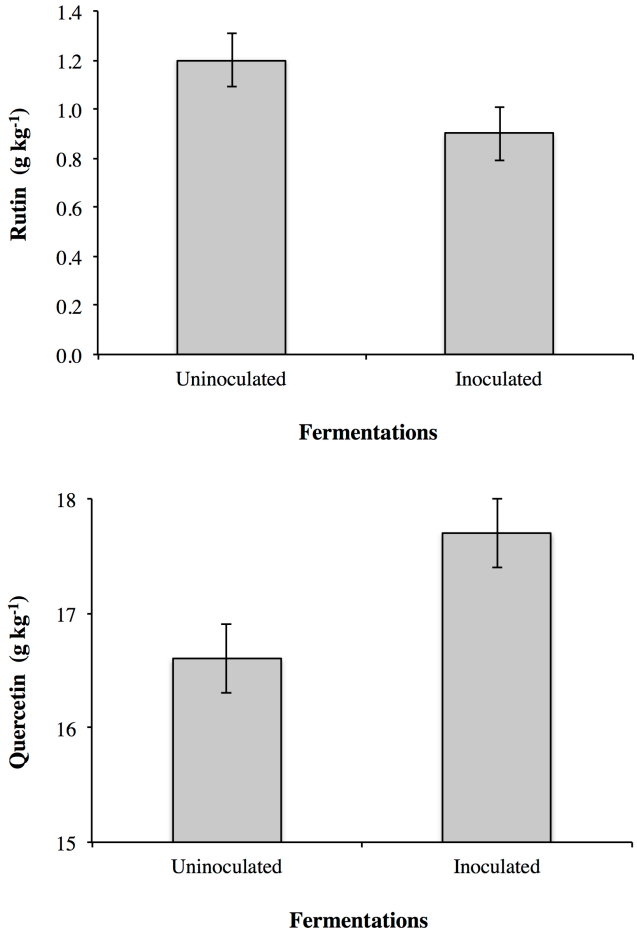
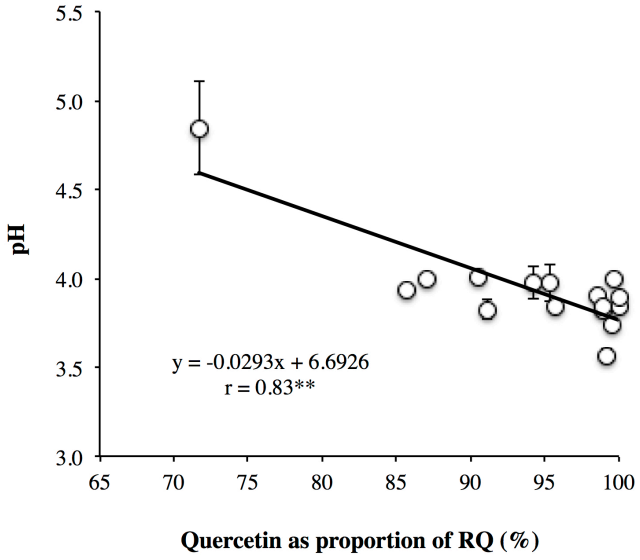


Figure 5 Relationship between pH and proportion of quercetin in the sum of rutin and quercetin concentration (RQ). Values are the mean of two years, and three replicates; vertical bars represent standard deviation of the mean, when not visible the error bar lies within the symbol.



Supporting information

Figure S1: Mariotti *et al.*

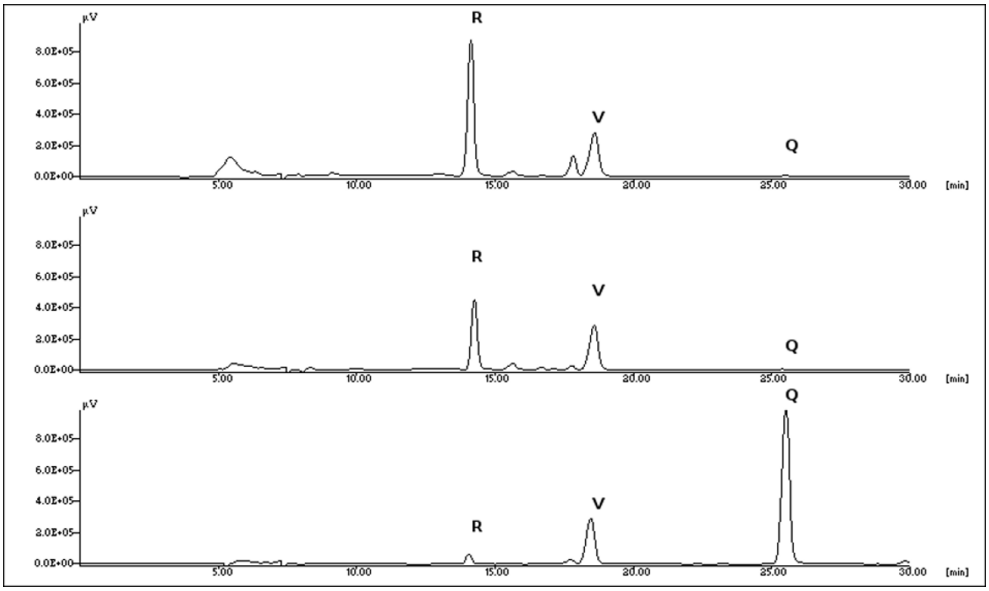


Figure S1 Sample HPLC chromatograms of fresh (top), hayed (middle) and ensiled (bottom) forage of common buckwheat. Peaks are identified as: R, rutin; V, vanillin; Q, quercetin.