RESEARCH

Isoflavone content in red clover (*Trifolium pratense* L.) as related to nitrogen and phosphorus application rate

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Red clover (*Trifolium pratense* L.) as a dietary supplement is currently used to treat menopausal symptoms because of its high concentrations of mildly estrogenic isoflavone. The application rate of N and P fertilizer has a huge influence on the isoflavone content (IC) in red clover. *Trifolium pratense* 'Minshan' is a unique plant resource in the alpine pasture region of northwest China. It plays a very important role in livestock production and the pharmaceutical industry because of its high nutritional value and IC. To date, little information exists on the selection of optimal N and P application rates to maximize the IC in red clover. Therefore, a 3-yr study was conducted to define the optimum N and P application rate to achieve a high IC in 'Minshan'. The results showed that topdressing 50 kg N ha^{-1} yr^{-1} with 52-105 kg P ha^{-1} yr^{-1} could produce a high level of IC at the branching, flower bud, early flowering, full flowering, pod setting, and mature stages in red clover. Second-year crops produced higher IC than did third-year crops. The IC was highest during the vegetative growing period and started to decline following the reproductive development of the plant until the seeds matured.

Key words: Fertilization rate, growth stage, isoflavone content, Minshan red clover.

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a perennial legume rich in isoflavone (Gu, 2007; Dabkeviciene et al., 2012). It has been used to prevent osteoporosis (Barnes, 2003), treat menopausal disorders (Beck et al., 2003) and protect from cardiovascular disease (Campbell et al., 2004). *Trifolium pratense* cv. Minshan has been a dominant cultivar for livestock production in the alpine pasture region of the Gansu province in northwest China for more than 50 yr. It has excellent cold and wet tolerance, adaptive capacity, and a high hay yield. In recent decades, it has become a very important isoflavone source for the pharmaceutical industry (Wang et al., 2005; Dabkeviciene et al., 2012).

In the cultivation of 'Minshan', farmers continuously increase N and P application rate to increase hay yield (Cupic et al., 2007; Yue et al., 2007; Song et al., 2012). However, under these high levels of N and P application, the isoflavone content (IC) has dropped dramatically according to testing results provided by hay companies (Wang et al., 2005). This reduces the sale price per unit and the economic value of red clover. Furthermore, the hay companies rejected the biomass from 0-50 cm aboveground because of its low IC. The resulting taller stubble that remains in the field greatly affects the regeneration of plants and consequently limits the production of further stubble. This means the management of N and P application becomes a vital factor for isoflavone production in 'Minshan'. Rossiter and Beck (1966) studied the effect of phosphate supply on the IC in subterranean clover (*T. subterraneum* L.) in a greenhouse and Butler et al. (1967) studied the effect of N, P, and K fertilizers on the IC in the leaves of red clover seedlings (60 d after germination) in a greenhouse. Their results indicated that the IC in the two clovers was influenced by N and P but not by K. To date, there has been no research conducted to study the effects of N and P application rates on IC in red clover throughout all the development stages under field conditions. The objective of this research was to study the influences of N and P fertilization rate on the IC of 'Minshan' at different growth stages and determine the optimal application rate to obtain maximum IC content. These results will be important for developing isoflavone production in red clover.

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MATERIAL AND METHODS

Field conditions and experimental design

The experiment was carried out at Lanzhou (36°03' N, 103°52' E; 1650 m a.s.l.) in Gansu Province, northwest China. This is one of the most suitable areas for red clover growth and is characterized by a hot summer growing season and cold winter. The annual rainfall of 350 mm is predominantly in summer. The evapotranspiration is 1486 mm, and the average temperature is 7.9 °C. The frost-free period is 210 d. The soil type is Kastanozem (FAO, 2006) comprising 62.3% sand, 21.5% clay, and 16.2% silt loam, and containing 1.21% OM, 128 mg soluble N kg^{-1}, and 41 mg effective P kg^{-1}. The pH is 7.8 and the bulk density is 1.23 g cm^{-3}.

The trial was completely randomized, and consisted of four N and P fertilizing rates (kg ha^{-1} yr^{-1}) in three replicates. The rates of N were 0 (N_0), 50 (N_{50}), 100 (N_{100}) and 150 (N_{150}) in the form of urea (46% N), and the rates of P were 0 (P_0), 52 (P_{52}), 105 (P_{105}) and 157 (P_{157}) in the form of superphosphate (5.24% P). The plot size was 5 × 5 m with six planted rows separated by 30 cm. Material was sown at a rate of 2.25 g m^{-2} at a depth of 2 cm on 6 May 2009. Only one rate of N and P (75 kg N ha^{-1} yr^{-1}, 105 kg P ha^{-1} yr^{-1}) was applied totally in the form of urea and superphosphate to each plot before sowing to obtain uniform growing conditions in 2009. In 2010 and 2011, red clover plants were fertilized at the returning green stage following the application rate designed for the experiment.

'Minshan' is a perennial plant. It grew very slowly and remained in the vegetative stage in the first year. Therefore, the fertilizer trial was conducted in 2010 and 2011.

Collection of samples and analysis of IC

Sample collection was carried out in 2010 and 2011, by randomly choosing a 100 cm-long row from each plot at the branching, flower bud, pod setting, and mature stages. In each plot, materials were cut just above ground at each stage while 50% of plants produced branches, flower buds, pods and mature pods. The samples for the early and full flowering stages were collected when 20% and 80% of plants were flowering, respectively (Chen, 2001). All the materials were dried in the lab at 21 °C. A hammer mill was used to pulverize samples when the water content dropped to 30% of dry mass. Samples of 10 g were used to determine IC.

The analysis procedure reported by Chen et al. (2004) was followed while preparing the standard and testing solutions, identifying the maximum absorption wavelength and calculating the standard curve.

Preparation of standard and testing solution. The concentration of standard solution was 62.5 μg each of daidzein, genistein, biochanin A, and formononetin standard samples (Sigma, St. Louis, Illinois, USA) in 1 mL methanol.

Two grams of red clover hay were wrapped in a filter paper and placed in a Soxhlet extractor with 150 mL of 70% ethanol. Extraction was performed at 90 °C for 3 h in a water bath with three replicates. The total extraction volume was brought to 50 mL with 70% ethanol and 5 mL of the ethanol extract was then transferred into a 10 mL sealed hydrolysis tube, mixed with 2 mL of 37% HCl solution, and hydrolyzed for 2 h at 83 °C in a water bath. The volume was brought up to 10 mL with 70% ethanol after the extract cooled to room temperature. The hydrolyzed extract was passed through a 0.45 μm nylon filter before performing HPLC analysis.

Preparation of standard curve and maximum absorption wavelength. Peak areas for 3, 6, 9, 12, 15 and 18 μL of standard solution were determined. Standard curves were generated with peak area and sample quantity as Y and X axis, respectively.

Absorption values of the standard solution at the wavelengths of 260 and 313 nm were analyzed following the procedure of Chen et al. (2004). The maximum value was obtained at the wavelength of 260 nm. This wavelength was thus selected for determining the IC in 'Minshan' in this study.

Measurement of IC

The IC was analyzed by HPLC according to the published procedures (Dornstauder et al., 2001; Delmonte et al., 2006; Khaosaad et al., 2008; Du et al., 2012). A 250 × 2.6 mm Lichrospher-C_{18} 5 μm column with a mobile phase consisting of acetonitrile and water was used. The gradient profile was acetonitrile increased from 20% to 70% in 20 min and the flow rate was 1 mL min^{-1} at room temperature. The isoflavone was detected at 260 nm. In each treatment, a 20 μL sample solution was used for HPLC analysis.

Data analysis

Data were analyzed statistically using the GLM procedure of SAS (SAS Institute, 2009). Duncan's Multiple Range Test (SAS Institute, 2009) was used to determine whether N or P fertilizing rate had significant influence on IC in 'Minshan'.

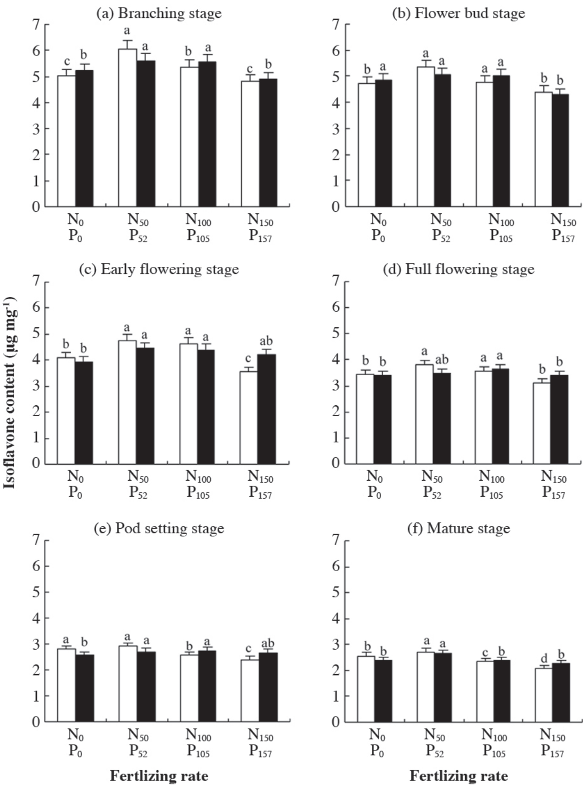
RESULTS

Branching stage

At this stage the IC varied with N and P fertilization rate, years and the N × P interaction (Table 1). As the N rate increased from 0 to 50 kg N ha^{-1} yr^{-1}, the IC increased from 5.03 to 6.07 μg mg^{-1}, then decreased to 4.83 μg mg^{-1} when N rate increased to 150 kg N ha^{-1} yr^{-1} (Figure 1a). The same tendency was observed with P fertilizer, as the P rate increased from 0 to 52 kg P ha^{-1} yr^{-1}, IC increased from 5.21 to 5.62 μg mg^{-1} and then dropped to 4.92 μg mg^{-1} when the P rate increased to 157 kg P ha^{-1} yr^{-1} (Figure 1a). Between the 2 yr, a higher IC (5.55 g mg^{-1}) was observed in 2010. The N × P interaction also had a great influence on IC and the highest value was obtained from $N_{50}P_{52}$, followed by $N_{50}P_{105}$, $N_{50}P_0$, and $N_{50}P_{157}$ (Table 2). The mean IC at this stage was the highest among the six growth stages.

Table 1. GLM results of N, P, year (Y), and the interaction between N and P (N×P) at different growth stages in 'Minshan' red clover.

	Branching stage		Flower bud stage		Early flowering stage		Full flowering stage		Pod setting stage		Mature stage	
Effect	F value	Significance	F value	Significance	F value	Significance	F value	Significance	F value	Significance	F value	Significance
N	24.64	< 0.0001	19.42	< 0.0001	29.62	< 0.0001	19.13	< 0.0001	27.64	< 0.0001	26.67	< 0.0001
P	8.61	< 0.0001	15.70	< 0.0001	5.09	< 0.01	3.39	< 0.05	2.37	> 0.05	9.72	< 0.0001
Y	17.47	< 0.0001	0.12	> 0.05	55.71	< 0.0001	14.74	< 0.01	921.34	< 0.0001	638.80	< 0.0001
N×P	3.20	< 0.01	3.49	< 0.01	0.56	> 0.05	3.13	< 0.01	2.61	< 0.05	5.87	< 0.0001



Bars on the same column diagram within the same growth stage represent standard errors and different letters indicate significant differences ($P < 0.05$).

Figure 1. Single effect of N and P application rate on isoflavone content in red clover at different growth stages (□: N; ■: P).

Flower bud and early flowering stage

The N and P fertilization rate and their interaction had a significant influence on IC at the flower bud stage, but not between the 2 yr (Table 1). The IC increased from 4.73 to 5.36 μg mg^{-1} when N rate increased from 0 to 50 kg N ha^{-1} yr^{-1}, but it decreased to 4.76 μg mg^{-1} when the N rate was > 50 kg N ha^{-1} yr^{-1} (Figure 1b). Among the P rates, the highest IC (5.08 μg mg^{-1}) was observed in P_{52}, followed by P_{105}, P_0, and P_{157} (Figure 1b). The N × P interaction had varying effects on the IC at this stage and the treatment $N_{50}P_{52}$ gave the highest value (Table 2).

Significant differences existed in the IC among different N and P fertilization rates, and between the years but not with the N × P interaction at the early flowering (Table 1). The highest IC (4.73 μg mg^{-1}) was obtained from N_{50}, followed by N_{100} (4.63 μg mg^{-1}), N_0 (4.08 μg mg^{-1}), and N_{150} (3.56 μg mg^{-1}) (Figure 1c). For P rates, the highest IC (4.46 μg mg^{-1}) was observed under P_{52}, followed by P_{105} (4.39 μg mg^{-1}), P_{157} (4.21 μg mg^{-1}), and P_0 (3.95 μg mg^{-1}) (Figure 1c). Red clover plants produced a higher IC in 2010 (4.63 μg mg^{-1}) than in 2011 (3.88 μg mg^{-1}). For the N × P interaction, the highest IC was produced from the $N_{50}P_{105}$ treatment (Table 2).

Full flowering stage

At this stage, significant differences occurred among the four factors, N rate, P rate, year, and the N × P interaction (Table 1). Among the different N rates, the highest value (3.79 μg mg^{-1}) was found in N_{50} (Figure 1d). For P rates, P_{105} gave the highest value (3.64 μg mg^{-1}) (Figure 1d). The IC varied between the 2 yr and the higher value (3.60 μg mg^{-1}) was observed in 2010. Treatment $N_{50}P_{105}$ produced the highest IC among the N × P interaction, followed by $N_{50}P_{52}$, $N_{50}P_0$, and $N_{50}P_{157}$ (Table 2).

Table 2. Effects of the interaction between N and P on isoflavone content (μg mg^{-1}) in red clover at different growth stages.

		P application			
Growth stage	N application	P_0	P_{52}	P_{105}	P_{157}
	kg N ha^{-1} yr^{-1}	kg P ha^{-1} yr^{-1}			
Branching stage	N_0	4.56d	5.10bcd	5.25bc	5.22bc
	N_{50}	5.65b	**6.71a**	6.28a	5.64b
	N_{100}	5.60bc	5.40bc	5.51bc	4.96cd
	N_{150}	5.04bcd	5.26bc	5.15bcd	3.88e
Flower bud stage	N_0	4.36de	4.94cd	4.87cde	4.77de
	N_{50}	5.32abc	**5.58a**	5.55ab	4.50cd
	N_{100}	5.05bcd	4.86cde	5.06bcd	4.05f
	N_{150}	4.66de	4.95cd	4.14ef	3.37g
Early flowering stage	N_0	3.71efg	4.17cdef	4.23cde	4.22cde
	N_{50}	4.44bcd	4.90ab	**5.00a**	4.61abc
	N_{100}	4.55abc	4.85ab	4.67abc	4.46abcd
	N_{150}	3.11h	3.91defg	3.65fgh	3.55gh
Full flowering stage	N_0	3.28cde	3.28cde	3.63bc	3.52bcd
	N_{50}	3.52bcd	4.04a	**4.06a**	3.52bcd
	N_{100}	3.74ab	3.71ab	3.57bcd	3.28cde
	N_{150}	3.01ef	2.89f	3.32cde	3.25def
Pod setting stage	N_0	2.90ab	2.69bcd	2.70bcd	2.88abc
	N_{50}	2.84abc	**2.97a**	2.96a	2.87abc
	N_{100}	2.31f	2.77abcd	2.64cde	2.59de
	N_{150}	2.26f	2.41ef	2.59de	2.32f
Mature stage	N_0	2.40cde	2.80ab	2.55bcde	2.50cde
	N_{50}	2.66abcd	**2.91a**	2.70abc	2.58bcde
	N_{100}	2.54bcde	2.39de	2.00f	2.46cde
	N_{150}	2.00f	2.51bcde	2.33e	1.51g

Different letters within a growth stage indicate significantly different values ($P < 0.05$).
Data are the means of the same treatment across 2 yr. The maximum values at the six growth stages are in bold.

Pod setting and mature stage

The mean IC at the pod setting stage was significantly lower than that of the other earlier stages. The N rates, years and N × P interaction had significant effects on IC but no difference occurred among the P rates (Table 1). Among the different N rates, the highest value (2.91 μg mg^{-1}) was obtained from N_{50} (Figure 1e). Red clover plants in 2010 also produced a higher IC (3.33 μg mg^{-1}) than in 2011 (2.01 μg mg^{-1}). From the N × P interaction, treatment $N_{50}P_{52}$ produced the highest IC at this stage (Table 2).

During the mature stage, red clover leaves turned yellow and started to drop, so the plants produced a very low IC (Table 2, Figure 1f). Significant differences in IC existed among N rates, P rates, years and the N × P interaction (Table 1). Among the different N rates, N_{50} produced the highest IC (2.71 μg mg^{-1}), followed by N_0, N_{100}, and N_{150} (Figure 1f). The same tendency was observed among the P rates (Figure 1f). 2010 produced a much higher IC (3.09 μg mg^{-1}) than that of 2011 (1.77 μg mg^{-1}). The N × P interaction had a strong influence on IC at this stage and the highest value was observed from treatment $N_{50}P_{52}$ (Table 2).

DISCUSSION

'Minshan' is one of the most important income providing crops for local farmers in the alpine pasture region of the Gansu Province in northwest China. Because of its high IC, it plays a vital role in animal husbandry and the pharmaceutical industry. Up to now, much of the research and farm management has focused on the improvement of hay production via fertilizer, irrigation, and cutting (Wang et al., 2005; Yue et al., 2007; Hu, 2009; Song et al., 2012). Studies indicated that IC in red clover was highly affected by environmental conditions (Schultz, 1971; Sivesind and Seguin, 2005; Du et al., 2012) and nutrition (Butler et al., 1967). The present study investigated the IC at different growth stages in 'Minshan' under various fertilizer applications. The results demonstrated that N and P fertilization rate had significant effects on the IC throughout all growth stages and a high IC could be achieved under appropriate N and P rates. Optimizing the application of fertilizer could greatly improve the isoflavone production and increase the economic value of red clover. This study provides important information for the management of red clover cropping to maximize isoflavone production through finding a balance between the IC and hay yield by optimizing fertilization.

Studies have shown that isoflavone levels in red clover plants were unaffected by K levels (Butler et al., 1967; Schultz, 1971). Therefore, K fertilizer was not considered in this study. It was found that P fertilizer had no effect on IC at the pod setting stage. However, across all other growth stages, either P or N fertilizer demonstrated significant effects on IC in 'Minshan' and higher values were obtained when N and P were applied at a low level (50 kg N ha^{-1} yr^{-1}, 52-105 kg P ha^{-1} yr^{-1}). A strong interaction between N and P fertilization rate existed in this study. The optimized application levels for achieving a high IC across most of the growth stages for 'Minshan' were 50 kg N ha^{-1} yr^{-1} plus 52 kg P ha^{-1} yr^{-1} or 50 kg N ha^{-1} yr^{-1} plus 105 kg P ha^{-1} yr^{-1}. These results were consistent with the related studies showing that higher levels of N and P reduced the IC level in subterranean clover and red clover (Rossiter and Beck, 1966; Butler et al., 1967).

In 2010 and 2011, the growth of red clover plants was slightly retarded by levels of N < 50 N ha^{-1} yr^{-1} mainly because the roots of plants became nodulated and appreciable quantities of atmospheric N were fixed as reported by Butler et al. (1967) and Rijke et al. (2005). A P rate > 52 kg P ha^{-1} yr^{-1} resulted in considerable retardation for most plants, which is also in agreement with a previous report by Rossiter and Beck (1966).

The IC decreased gradually with the maturation of the red clover plants. The main reason is that isoflavone is a secondary product of metabolism (Barnes, 2003; Dabkeviciene et al., 2012). Its production varied during the development of red clover plants (Du et al., 2012), and peaked during the vegetative growing period while the plants had the strongest functional activity for photosynthesis and metabolism (Gu, 2007).

The life span of red clover is 5-6 yr. 'Minshan' has the strongest vigor and growth condition in its second year. From the third year, some branches start to die. Therefore, IC in the second year plants was significantly higher than in those of the third year. Apart from this, the powdery mildew (*Uncinula bicornis* (Wallr. ex Fr.) Lév.) was a contributor to IC decline in the third year. Field investigations indicated that powdery mildew was much more severe in third year plants and that powdery mildew infected tissue had a lower IC than that of the healthy parts (unpublished data).

CONCLUSION

The optimized application levels for achieving a high IC across most of the growth stages for 'Minshan' red clover were 50 kg N ha^{-1} yr^{-1} plus 52 kg P ha^{-1} yr^{-1} or 50kg N ha^{-1} yr^{-1} plus 105 kg P ha^{-1} yr^{-1}.

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