Environmental advantages of binary mixtures of *Trifolium incarnatum* and *Lolium multiflorum* over individual pure stands

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ABSTRACT

To investigate the environmental advantages of using grass-clover binary mixtures over pure stands as winter cover crops, a serial of five field experiments (each designed as randomized complete blocks with four replicates) was carried out in eastern Slovenia. *Trifolium incarnatum* L. and *Lolium multiflorum* Lam. were sown in late summer as pure stands and binary mixtures. Pooled data calculated from all the experiments revealed that the soil mineral N in spring and accumulation of N by plants decreased with decreasing proportion of *T. incarnatum* in the binary mixtures, while the C:N ratio of cover crop organic matter increased. C accumulation was the highest when the seeding ratio of the binary mixture of *T. incarnatum* and *L. multiflorum* was 50:50. In the C and N environmentally sustainable management efficiency coefficients, three important traits of winter cover crops for environmental protection were given equal importance (low soil mineral N content in spring, high C accumulation in plants, and high N accumulation in plants). The coefficient was higher for binary mixtures of *T. incarnatum* and *L. multiflorum* than for pure stands of these crops, proving the complex environmental advantages of binary mixtures over pure stands.

Keywords: carbon accumulation; cover crops; field rotation; nitrogen accumulation; soil mineral nitrogen

Increased environmental awareness over the past few decades has challenged farmers to pursue optimal C and N management in field rotation. With C sequestration (Reicosky and Forcela 1998, Sainju et al. 2008), winter cover crops mitigate greenhouse CO_2 emissions and increase soil organic C dynamics. Similarly, N sequestration by winter cover crops reduces N leaching, greenhouse gas N_2O emission, and (in particular in a case of symbiotically active legumes) reduces the rate and cost of N fertilization (Sainju et al. 2008).

Extensive research has been carried out to investigate winter cover crops cultivated in pure stands, particularly over the past couple of years (e.g. Kramberger et al. 2009, Thorup-Kristensen and Dresbøll 2010, Campiglia et al. 2011, Salmerón et al. 2011). However, many researchers have studied mixtures of leguminous and non-leguminous winter cover crops in anticipation of the agricultural or ecological advantages of such binary mixtures (e.g. Möller et al. 2008, Brennan et al. 2011, Ćupina et al. 2011). Nevertheless, the environmental advantages of mixed cultures over pure cultures are still not adequately described in the complex way taking more parameters into one account.

Trifolium incarnatum and *Lolium multiflorum* are worldwide sown through temperate areas (in pure stands and in mixtures) as summer (Brant et al. 2009) or usually as winter catch crops for forage or as green manure crops (winter cover crops) in the field rotation. Therefore, this research aimed to assess the C and N management-related complex advantages of binary mixtures of the winter cover crops *T. incarnatum* and *L. multiflorum* over their pure stands in terms of environmental expectations in field rotation.

MATERIAL AND METHODS

Experimental sites and setup. From 2008–2011, we carried out five field experiments in the low-

Table 1. Total monthly weather parameters and their long-term average values during the experimental period**

Month	Precipitation (mm)					Temperature (°C)				
	2008	2009	2010	2011	LTA*	2008	2009	2010	2011	LTA*
January	–	107	35	4	42	–	–1.5	–1.6	1.0	0.2
February	–	49	49	1	36	–	1.9	1.4	0.5	2.2
March	–	67	25	31	62	–	6.5	6.1	6.5	6.4
April	–	42	54	23	55	–	13.7	11.2	13.2	11.3
May	–	130	73	62	83	–	17.1	15.7	16.4	16.6
June	96	165	86	–	112	20.2	18.5	20.1	–	20.1
July	110	127	76	–	102	21.3	21.5	23.0	–	21.9
August	134	284	153	–	141	20.7	21.2	17.7	–	20.5
September	61	103	136	–	129	14.9	17.1	14.0	–	15.3
October	44	46	36	–	61	11.6	10.6	8.6	–	10.7
November	46	58	121	–	57	6.2	6.9	6.6	–	6.1
December	102	55	21	–	55	1.9	1.9	0.3	–	0.8

*long-term average; **experiment I: August 2008 to May 2009; experiment II: August 2008 to May 2009; Experiment III: August 2009 to May 2010; Experiment IV: August 2009 to May 2010; and Experiment V: August 2010 to May 2011

land area south of Maribor, Slovenia. The weather characteristics of the region (Grm and Kuzma 2011) during the experimental period are presented in Table 1 and the site and soil characteristics for individual experiments in Table 2.

Each experiment (Table 2) was conducted in a randomized complete block design with four replicates. The plot size was 30 m × 18 m. Winter wheat was the previous crop for all the experiments, and the straw was removed from the field in all cases. At the end of August, *Trifolium incarnatum* L. cv. Inkara and *Lolium multiflorum* Lam. ssp. *italicum* Shinz and Kell. cv. Lipo were seeded in pure stands (at 30 and 50 kg/ha, respectively) and as binary mixtures (seeding ratios 75:25; 50:50; 25:75; i.e. 22.5 kg *T. incarnatum* + 12.5 kg *L. multiflorum*, 15.0 kg *T. incarnatum* + 25.0 kg *L. multiflorum*, 7.5 kg *T. incarnatum* + 37.5 kg *L. multiflorum* per

Table 2. Site and soil characteristics

	Experiment				
	I	II	III	IV	V
Site	46°30'40''N 15°40'30''E 269 m a.s.l.	46°29'50''N 15°37'50''E 280 m a.s.l.	46°29'40''N 15°40'20''E 265 m a.s.l.	46°30'10''N 15°37'50''E 285 m a.s.l.	46°30'20''N 15°39'50''E 268 m a.s.l.
Soil (FAO classification)	Dystric cambisol (shallow)	Dystric cambisol (deep)	Dystric cambisol (shallow)	Dystric cambisol (deep)	Dystric cambisol (shallow)
Soil pH (0.1 mol/L KCl)	6.1	5.8	5.8	5.9	7.0
Soluble P – ammonlactate extraction procedure (Egnér et al. 1960) (g/kg of air dry soil of ploughing layer)	0.077	0.126	0.176	0.111	0.174
Soluble K – ammonlactate extraction procedure (Egnér et al. 1960) (g/kg of air dry soil of ploughing layer)	0.219	0.173	0.368	0.236	0.440
Soil N_{min} before cover crop seeding (kg/ha)	66.4	65.4	28.9	22.3	88.8

hectare). Experiments included treatment of field without any cover crop (bare fallow). No organic or mineral fertilizers were used for fertilising the cover crops.

Measurements and analytical determinations. To measure the soil mineral nitrogen (N_{min}) in the 0–60-cm soil layer before cover crop seeding in August and at the end of April next year, ten sub-samples per plot were obtained using a soil probe (diameter: 4 cm), mixed, sub-sampled, and frozen immediately after collection. Later, the soil sub-samples were analysed for ammonium-N and nitrate-N (N_{min}) according to the method described by Keeney and Nelson (1982).

At the end of April, the aboveground biomass yield of the cover crops and weeds in bare fallow was obtained by cutting the plants to a height of 5 cm from six quadrats (50 × 50 cm) of each plot and weighing them. The yield of the remaining stems and roots was measured by collecting them from two 40 × 40 cm^2 quadrats in each plot. The aliquot sub-samples (approximately 1.5 kg each) of the cover crops and weed were used to determine dry matter yields (DM) by drying to constant mass at 70°C in a forced-draught oven. The N and C contents of the cover crops and weeds were measured using the Kjeldahl method and the Walkley and Black's method, respectively (Nelson and Summers 1996), assuming that all plant C was oxidised during digestion.

The coefficient of C and N environmentally sustainable management efficiency (CCNEME) of the observed cover crop treatments was calculated using formula:

$$CCNEME_{itreat} = 1/3(N_{ityield}/N_{eyieldaver} + C_{ityield}/C_{eyieldaver} - N_{itmin}/N_{eminaver})$$

Where: $CCNEME_{itreat}$ – C and N environmentally sustainable management efficiency of individual treatments; $N_{ityield}$ – amount of N accumulated in individual treatment; $N_{eyieldaver}$ – average amount of N accumulated in the experiment; $C_{ityield}$ – amount of C accumulated in the individual treatment; $C_{eyieldaver}$ – average amount of N accumulated in the experiment; N_{itmin} – amount of soil mineral $N_{(0-60\ cm)}$ of individual treatments in spring, and $N_{eminaver}$ – average amount of soil mineral $N_{(0-60\ cm)}$ of experimental treatments in spring.

For calculating the CCNEME from data obtained from other studies (Table 4) where the plant C content was not analysed, the plant C concentration was estimated to be 40% of the DM, as used in the study of Hanly and Gregg (2004).

Statistical analyses. Aiming to provide easer interpretation of the results and statistical significance, data from all studies were polled into one (six treatments with 20 replicates), which was possible because of the same experimental setups, measurements and analytical determinations. The data of all investigated variables were subjected to an analysis of variance (ANOVA). The mean separation among treatments was obtained using the Fisher's *LSD* test. Statistical significance was evaluated at $P \leq 0.05$.

RESULTS

The different cover crop treatments significantly affected the soil N_{min} content in spring (Figure 1). Pure stand of *L. multiflorum* as well as its binary mixtures with *T. incarnatum* (75% and 50% ratios of *L. multiflorum*) resulted in low soil $N_{min\ (0-60\ cm)}$ content. On the contrary, pure stand of *T. incarnatum* resulted in high soil $N_{min\ (0-60\ cm)}$ content.

Table 3. Effect of treatment on the amount of N, C accumulation in plants and C:N ratio across different cover crops

Treatment	Accumulated N in plants (kg/ha)	Accumulated C in plants (kg/ha)	C:N ratio
*T. incar** (100%)	135.1^{a}	2221.3^{bc}	16.0^{c}
T. incar (75%) + *L. mult* (25%)	105.5^{bc}	2521.5^{ab}	24.4^{b}
T. incar (50%) + *L. mult* (50%)	109.4^{b}	2821.1^{a}	26.2^{b}
T. incar (25%) + *L. mult* (75%)	90.5^{c}	2530.7^{ab}	28.6^{b}
L. mult (100%)	51.1^{d}	2085.8^{c}	45.5^{a}
Bare fallow	31.8^{e}	972.5^{d}	26.9^{b}

**T. incar* – *Trifolium incarnatum*; *L. mult* – *Lolium multiflorum*. Means followed by different letters in the same column are significantly different ($P \leq 0.05$, Fisher's *LSD* test)

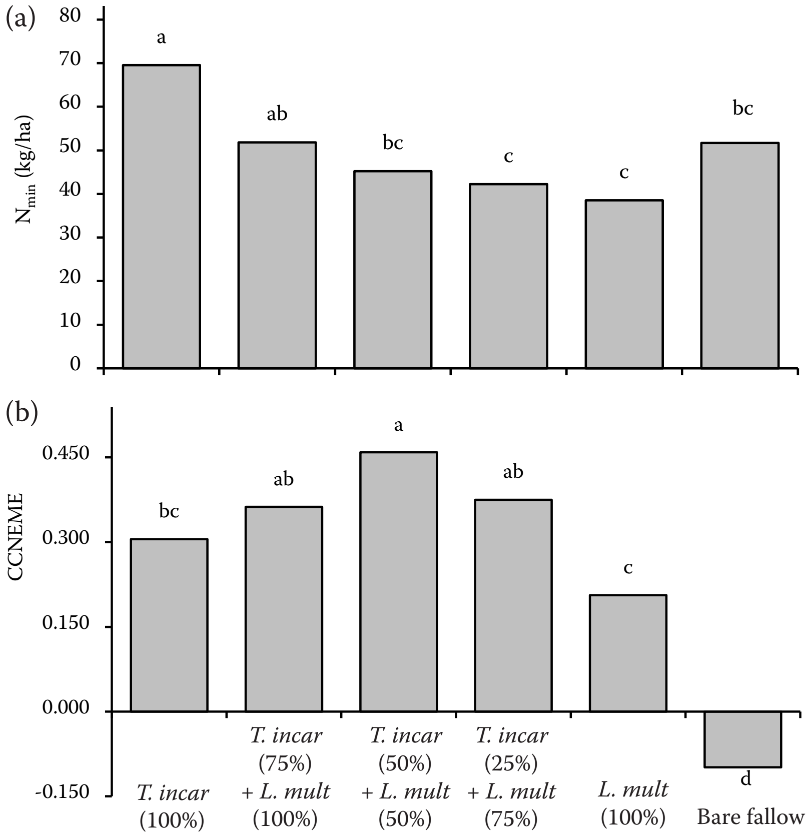

Figure 1. (a) The effect of winter cover crops on the soil N_{min} content in spring (The average soil $N_{min(0–60\ cm)}$ at the beginning of the experimental period was 54.4 kg/ha) and (b) The coefficients of C and N environmentally sustainable management efficiency (CCNEME). *T. incar* – *Trifolium incarnatum*; *L. mult* – *Lolium multiflorum*. Bars followed by different letters are significantly different ($P \leq 0.05$, Fisher's *LSD* test)

Further, bare fallow had significantly lower soil $N_{min\ (0–60\ cm)}$ content than pure stand of *T. incarnatum*.

Pure stand of *T. incarnatum* (Table 3) showed the highest amount of N accumulation in cover crops (aboveground biomass plus roots), which decreased with the increasing proportion of *L. multiflorum* in the binary mixtures. Pure stand of *L. multiflorum* showed the lowest N accumulation.

These results indicate that the cover crop treatments significantly affected C accumulation in plants (aboveground biomass plus roots). The binary mixture of equal proportions of *T. incarnatum* and *L. multiflorum* (50% each) accumulated higher C levels than the pure stands of *T. incarnatum* or *L. multiflorum* alone (Table 3). The amount of accumulated C did not differ significantly across the mixtures and the *T. incarnatum* pure stand. Weeds on bare fallow accumulated only 972.5 kg/ha. The highest C:N ratio of organic matter was obtained in pure stand of *L. multiflorum*, and the lowest in pure stand of *T. incarnatum* (Table 3).

The coefficient of C and N environmentally sustainable management efficiency (CCNEME) for *T. incarnatum* and *L. multiflorum* binary stand

Table 4. The coefficients of C and N environmentally sustainable management efficiency (CCNEME) for winter cover crops in pure stands and binary mixtures for data from studies of other authors

Treatment	CCNEME				Details	Data source
	F	A	B	AB		
F Fallow, A *Secale cereale*, B *Vicia villosa*	–0.17	0.48	0.47	0.57	Average over two pre-cover crop treatments and over two years	Ranells and Wagger (1997)
F Fallow, A *Secale cereale*, B *Trifolium incarnatum*	–0.28	0.48	0.53	0.62		
A *Secale cereale*, B *Vicia villosa*	–	0.29	0.16	0.54	CCNEME for 1995; in 1996 *V. villosa* pure standsdid not survive the winter. No data available for fallow biomass.	Vaughan et al. (2000)
A *Triticum aestivum*, B *Vicia villosa*	–	0.35	0.12	0.52		

with a 50:50 ratio reached 0.46, and was statistically higher than the corresponding value for pure stands of *T. incarnatum* and *L. multiflorum*. The CCNEME values for binary mixtures containing a higher proportion of one plant species were between two statistical extremes and differed only from the values of *L. multiflorum* pure stand. As shown in Figure 1, the CCNEME value for bare fallow covered with weeds was negative.

Thus far, only two scientific articles (Ranells and Wagger 1996, Vaughan et al. 2000) in literature have studied leguminous and non-leguminous winter cover crops grown as pure stands and binary mixtures and reported data required for CCNEME calculations. In both these studies (Table 4), the CCNEME values of winter cover crops were higher in binary mixtures than in pure stands.

DISCUSSION

The results of soil N_{min} content of *T. incarnatum* and *L. multiflorum* pure stands in spring obtained in our experiment (Figure 1) support earlier findings (Thorup-Kristensen 2001, Kramberger et al. 2009, Plaza et al. 2011), which report that the soil N_{min} of non-leguminous cover crops is lower than that of symbiotically active leguminous cover crops. Consequently, in areas with high rainfall during autumn, winter, and spring, symbiotically active leguminous winter cover crops increase the risk of higher nitrate leaching. Despite the fact that binary mixtures (Figure 1), particularly mixtures with high proportion of *T. incarnatum*, have higher soil N_{min} content than pure stands of *L. multiflorum*, it is recommendable to use mixtures than pure stands of *T. incarnatum* in cases where there is high risk of nitrogen leaving the system.

Considering that the straw of previous winter wheat crops was removed from the field and cover crops were not fertilised with N, the accumulated N levels in the observed cover crops reached expected values with the highest level observed in pure stands of *T. incarnatum* and the lowest, in pure stands of *L. multiflorum* (Table 3). Similar results were obtained in other studies (Rannels and Wagger 1996, Hanly and Gregg 2004, Gabriel and Quemada 2011) but not in the studies where maize residues (maize was intensively fertilised with N) were chopped and left on the field (Salmerón et al. 2011). The amount of N accumulated in non-leguminous cover crops namely depends on the available soil N_{min}, while in the case of symbiotically active legumes, the majority of N accumulated in individual plants mainly occurs due to N fixation.

The binary mixture of *T. incarnatum* and *L. multiflorum* (50% each; Table 3) showed higher C accumulation than pure stands of *T. incarnatum* and *L. multiflorum*; this finding can be easily explained by higher dry matter yield of the cover crop biomass, which can be result of more effective N symbiosis and C fixation by legumes in such mixtures (Schipanski et al. 2010). Similar results of pure stands of the cover crops and their mixtures yields were obtained in many studies (Rannels and Wagger 1996, Vaughan et al. 2000, Kuo and Jellum 2002), while some studies reported the dry matter yield of binary mixtures to be between the yields of the individual pure stands (Rannels and Wagger 1996, Hanly and Gregg 2004) or at least at the same level as that of the pure stand of the less productive crop (Ćupina et al. 2011). This variation in the results can be explained by ecological concepts of niche differentiation. Higher productivity of mixtures relative to that of pure stands occurs if the between-species competition is low, and complementarity between species promotes better use of natural resources (Hill and Shimamoto 1973, Snaydon 1991, Möller et al. 2008). The yield advantage of binary mixtures over pure stands can be expected when suitable components in the mixture are brought together for specific growing conditions. The good growth of all the components of the mixture is not important only for a better yield as compared to the pure strand but also for the environmental protection by the cover crop such as low soil N_{min} content, C and N accumulation, symbiotic N fixation, and C:N ratio. However, field rotation, appropriate cover crop residue management, synchronisation of CO_2 and inorganic N release by mineralisation of cover crop residues are necessary to combine with demands by the subsequent crops in field rotation for achieving all these environmental benefits with cover crops. Here, the C:N ratio of cover crop organic matter plays an important role; residues of symbiotically active legumes may have low C:N ratio and faster mineralisation than non-leguminous residues with higher C:N ratios (Rannels and Wagger 1996). As shown in Table 3, with the use of mixtures of leguminous and non-leguminous crops, the C:N ratio can be planned according to the requirements of the subsequent crops in field rotation.

For highlighting the advantages of mixtures compared to cover crop pure stands in term of environmental protection, the coefficient of C and N environmentally sustainable management efficiency (CCNEME) was created. According to the data from our experiments, the higher CCNEME value for *T. incarnatum*/*L. multiflorum* binary mixtures compared to their pure stands counterparts indicate the overall environmental advantages of the binary mixtures (Figure 1). On the basis of the available results, the CCNEME calculated from the data of other studies was found to be higher for leguminous and non-leguminous binary mixtures than for pure stands of the individual crops (Table 4), supporting our findings and emphasising on the importance of their agricultural and ecological advantages as reported previously (e.g. Möller et al. 2008, Brennan et al. 2011). Despite the accumulation of certain amount of C and N by annual weeds (Table 3), the CCNEME of bare fallow was negative (Figure 1), proving the environmental advantages of all the cover crops used in our experiments, including the pure stands of non-leguminous *L. multiflorum*.

From the environmental protection perspective, binary mixtures of leguminous and non-leguminous winter cover crops can provide many advantages over their pure stands. Their benefits arise mostly from their complex efficiency in C and N management. However, to achieve the benefits, it is extremely important that the leguminous and non-leguminous winter cover crop mixtures be composed of complementary components that will facilitate good growth under field-specific growing conditions.

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