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Winter Pea Mixtures with Triticale and Oat for Biogas and Methane Production in Semiarid Conditions of the South Pannonian Basin

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Abstract: Due to the increase in greenhouse gases from burning fossil fuels, there is increased attention on renewable energy sources from specialized crops. These crops should not compete with food security, and it is important to select plant resources which can produce methane-rich biogas efficiently. The most commonly used energy crops are planted and managed intensively with high inputs in productive land, and this negatively affects land use and sustainable use of resources. The main purposes of this study are to: (a) determine the best cropping system for optimal biogas and methane production from sole crops of winter pea, triticale and out and their mixtures at two different maturity stages (first stage: full-flowering stage of winter pea and beginning of milky stage of cereals; second stage: emergence of firsts pods for pea and milky/waxy stage of cereals); and (b) to develop and use a surface model to determine the best combinations of various mixtures that result in highest biogas and methane. The used pure or mixtures of pea, oat and triticale in two seed weight ratios (50%:50% and 75%:25%) produced different green mass, dry matter, solids, biogas and methane yields. The experiments showed that maximum green mass was produced by the mixture of pea and oat at the seed ratio 75%:25% and when crop was harvested at the full-flowering stage of winter pea and beginning of the milky stage of cereals. After quadratic model analyses, the combination ratios of the oat and triticale were, respectively, 30% and 8%, with a maximum green biomass yield of 61.48 t ha^{-1}, while the corresponding values were 28% and 38%, with maximum solids yields of 25.64 t ha^{-1}. As the model was set at 100 for all three independent variables (oat, triticale and pea), the pea should be at 62% (100-30-8) and 34% (100-28-38), respectively, for green mass and organic solids yields. The results of surface analysis and multivariate analysis of variance showed that the mixture of oat and triticale had great potentiality for biogas and methane yields. The optimal mixture of oat with triticale was 27~35% with 73~65% for producing biogas and (or) methane.

Keywords: crops mixtures; biogas; methane production; semiarid conditions

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1. Introduction

Climate change caused by anthropogenic greenhouse gas (GHG) emissions is a major global challenge that needs immediate attention. At the present, fossil fuels (coal, oil and natural gas) are the primary sources of energy. Combustion of fossil fuels and related processes have contributed to largest proportion of the total GHGs over the last several decades, with an annual production of more than 30 billion tonnes of carbon dioxide (CO_2) [1]. There have been several measures at the global level to minimize dependence on fossil fuels [2] and find alternatives sources of energy. One of these measures is to explore and use renewable energy sources from crop and residues. Although there has been research on use of grain crops for biofuels, the use of such crops competes with food security. To minimize the competition between food grains and energy production, using lignocellulosic biomass through anaerobic digestion process is a good option. Production of methane-rich biogas through anaerobic digestion of organic materials has been evaluated as one of the most energy efficient and environmentally benign pathways of renewable energy generation [3]. The special importance of the production and use of biogas is to prevent emission of methane that increases the greenhouse effect (the intensity is 23 times greater than CO_2) [4].

The concept to use dedicated plant biomass, the so called "bioenergy crops" for methane production (bio methanation) is not new. Early investigations on the bio methanation potential of different crops and plant materials were carried out in the 1930s by Buswell in the USA and later on in the 1950s by Reinhold and Noack in Germany [5]. The potential use of oats (*Avena sativa* L.), grass and straw was demonstrated in New Zealand, resulting in methane yields of 170–280 $m^3\ t^{-1}$ total solids (TS) [6]. Even water hyacinths and freshwater algae were shown to result methane yields between 150 and 240 $m^3\ t^{-1}$ total solids (TS) [7]. In the USA, a large project on microalgae and kelp for aquatic raw material production was initiated [8]. Although the digestion of crop material was demonstrated, the process was hardly applied in practice [9]. Crop digestion was commonly not considered to be economically feasible. Crops, plants, plant by-products and waste materials were just added occasionally to stabilize anaerobic waste digesters [10]. With steadily increasing oil prices and the need for a climate framework, "bioenergy crop" research and development gained attention in the 1990s. In Germany, for example, the number of digesters using energy crops has increased from about 100 in 1990 to nearly 8000 in 2014 [11]. To renew this Paris Climate Agreement was signed to curb greenhouse gases and keep global temperature rise below 2 °C and its implementation has gained global attention [12].

Many conventional forage crops are easy to cultivate and produce large amount of biomass. Furthermore, being bred for animal feed, these crops are often characterized by good digestibility [13]. The most important parameter in choosing crops for methane production is the net energy yield per hectare, which is defined mainly by biomass yield and convertibility of the biomass to methane [14]. Continuous production of homogeneous biomass is crucial for a constant supply of digester. Currently, most bioenergy crops (as well as most important food crops) are grown as intensive monocultures. In Germany, nearly 80% of all energy crops for biogas production are maize (*Zea mays* L.) for silage [15]. This dominance of monocropping system frequently causes ecological problems such as loss of biodiversity, soil erosion, nitrogen leaching and increased use of pesticides due to enhanced pressure of pests and diseases in tight crop rotations or monocultures [16]. In order to meet these targets, the double cropping system has been suggested and intensively investigated over recent years [17]. It involves the cultivation of two crops during the first year with the combination of winter-hardy crops (first crop) and summer crops (second crop).

The Pannonian basin is one of the largest regions in Europe which extends over nine countries including Serbia. This region has been sensitive to climate change and is increasingly confronted with heat waves and drought [18,19]. Finding alternatives fuel sources, especially the crops that are already grown in the region, would be important. Traditionally, in the Republic of Serbia, mixtures of winter pea (*Pisum sativum* subsp. *arvense* L.) with winter cereals (triticale × *Triticosecale*), and oat, *Avena sativa* L.) are used extensively for forage production as the first crops in double cropping system, which involves, as the second crops, maize and sorghum (*Sorghum bicolor* L. Moench) for silage. Leguminous crops form root nodules with the ability to bind nitrogen from the atmosphere. Thus, they require little fertilization and contribute to efficient turnover of nitrogen in agriculture [20]. Methane production of a specific crop is affected by the chemical composition of the plant which changes as the plant matures [21,22], and timing and frequency of harvest are thus critical in order to optimize the biomass yield and feedstock quality [23].

At the same time, based on our previous study [24], it was clear that mixtures of winter pea with triticale at the seeding ratio (50:50) at full-flowering stage of winter pea achieved a higher biogas and methane yield per t of volatile solids than the other different mixtures.

Our previous studies showed that use of crop mixtures such as winter pea with either oat or triticale were more productive and profitable compared to monocropping systems. In addition, it was observed that a seed ratio of 85:15 (pea and cereals) had the biggest advantage from intercropping, which was attributed to greater land use efficiency [25]. Our preliminary research showed that these mixtures can be a good source of biofuel production [24]. However, the crop ratios that have the best biomass production may not be the same for biomass production and for biogas and methane. This is because the crop stage of development can have a significant influence on total biomass production and traits such has quantities of volatile solids that are key for biofuel production.

The main purpose of this study was to (a) determine the best cropping system for optimal biogas and methane production from sole crops of winter pea, triticale and out and their mixtures at two different maturity stages (full-flowering stage of winter pea and milky/waxy stage of cereals); and (b) to develop and use a surface model to determine the best combinations of various mixtures that result in the highest biogas and methane yields. We hypothesize that the optimum mixtures (proportions) of pea and cereals will be different for green biomass, biogas or methane production.

2. Materials and Methods

2.1. Field Experiment

The experiments were carried out during 2012–2013 and 2013–2014 growing seasons at the Institute of Field and Vegetable Crops in Novi Sad, Serbia, on a field characterized as chernozem soil. The experimental site is located at N 45°26′82″ E 19°83′18″, in Vojvodina of Serbia. The soil at the experimental site was slightly alkaline with a pH range from 7.18 to 8.25, and well supplied with phosphorus and potassium; the humus content ranged from 2.58 to 3.52 (Table 1). The previous crop was spring vetch in both years.

Table 1. Chemical properties of the soil.

Year	pH		Humus (%)	Total Nitrogen (%)	mg 100 g Soil	
	in H_2O	in KCl			P_2O_5	K_2O
2012	8.10	7.18	3.52	0.241	61.90	40.0
2013	8.25	7.28	2.58	0.19	32.4	30.6

Weather data during the growing seasons (October to May) are given in Table 2. The conditions (temperature and precipitation) during the autumn period in both years (2012–2013) were favorable for sowing and establishment of winter crops until the end of October (Table 2, Figure S1).

Table 2. Precipitation and mean monthly temperature for growing season (2012–2014) and long-term average (1963–2013) at the experiment site.

Month	Precipitation (mm)			Temperature (°C)		
	2012–2013	2013–2014	1963–2013	2012–2013	2013–2014	1963–2013
October	49	67	45	13.7	14.7	11.7
November	36	41	52	10.0	9.03	6.0
December	55	2	46	1.0	2.0	1.7
January	60	24	37	3.0	4.3	·0.3
February	49	10	33	4.3	7.0	1.7
March	68	50	39	6.0	10.4	6.3
April	30	49	47	13.7	13.4	11.7
May	118	203	60	18.4	16.4	17.0
Total	465	446	359			
Average				8.7	9.6	7.0

Winter pea, oat and triticale monocultures as well as mixtures of winter pea with both cereals, in two seed weight ratios (50:50 and 75:25) were sown on 26 October 2012 and 29 October 2013, respectively (Table 3). Seed weight ratios were based on the original full mixture of the respective pure crops. In all plots, crops were mechanically planted. The row spacing was 12.5 cm and the experimental design was a randomized complete block with seven treatments—three monocultures and four mixtures of winter pea with cereals replicated four times. Individual plot size for each treatment was 5 m × 20 m.

Table 3. Treatment details of the field experiments. Each treatment was harvested at two different maturity stages (1st stage: full-flowering stage of winter pea and beginning of milky stage of cereals; 2nd stage: emergence of firsts pods for pea and milky/waxy stage of cereals).

Treatment	Composition of Crops	Seed Weight Ratios
1.	Pure crops of oat	100% (150 kg ha^{-1})
2.	Winter pea: oat mixture	50%:50% (70 + 75 kg ha^{-1})
3.	Winter pea: oat mixture	75%:25% (105 + 37.5 kg ha^{-1})
4.	Pure crops of pea	100% (140 kg ha^{-1})
5.	Pure crops of triticale	100% (250 kg ha^{-1})
6.	Winter pea: triticale mixture	50%:50% (70 + 125 kg ha^{-1})
7.	Winter pea: triticale mixture	75%:25% (105 + 62.5 kg ha^{-1})

Seed weight ratios based on the original full mixture of the respective pure crops.

Harvesting dates of pure crops of pea and cereals as well as all of mixtures at the first maturity stage were 27 May 2013 and 20 May 2014, respectively. When it comes to the second maturity stage, harvesting dates for all treatments were 18 June 2013 and 2 June 2014. Each treatment group was harvested at two different maturity stages generally corresponding to: 1. full-flowering stage of winter pea and 2. milky/waxy stage of cereals. For reasonable statistical analyses, we theoretically transformed the portions of the three factors, oat (*X*1), pea (*X*2) and triticale (*X*3), at a 0.95 confidence interval in a supplementary table (Table S1).

2.2. Chemical Analysis and Calculation of Methane Yield

For determination of the dry matter yield, land area of 1 m^2 was harvested in the center of each plot at two maturity stages (1st stage: full-flowering stage of winter pea and beginning of milky stage of cereals; 2nd stage: emergence of first pods for pea and milky/waxy stage of cereals). Samples taken from the plots were chopped to approximately 1 cm size and dried in oven at 60 °C for 72 h to determine dry weight. A subsample of approx. 1 kg was taken and dried at 60 °C in a drying cabinet to constant weight to establish the dry matter (DM) content. The dried subsample was milled in a cutting mill SM 200 (Retsch, Haan) using a 1 mm sieve for chemical and biogas analyses. All samples were investigated for potential biogas yield [26] and methane content in batches using 1 L glass bottles in controlled climate chambers [27]. Temperature mode was mesophilic (38 °C), and the samples were inoculated with the digestate from operating biogas plant in the vicinity, which uses cattle manure and maize silage as substrate. The cow manure was obtained from a dairy farm in Čenej. Biogas samples were taken with a pressure lock syringe and their methane content was measured with gas chromatographs (GCs) equipped with a flame ionization detector.

Total solids (TS) and volatile solids (VS) were determined according to the standard methods [28]. Methane potentials of substrates were calculated as m^3 CH_4 kg^{-1} VS_{added}, m^3 CH_4 kg^{-1} TS_{added} and m^3 CH_4 t^{-1} ww, minus the methane potential of the inoculum.

Methane production is reported in normalized liter per gram of volatile solids of energy crop [$_N$L CH_4 (g VS)$^{-1}$], i.e., the volume of methane production was standardized on specific temperature and pressure conditions (273 K; 1 atm) [27]. Methane production from the inoculum alone was measured and subtracted from the methane production as background noise in digested crop bioreactors.

2.3. Data Analysis and Statistical Methods

Individual and combined data for both years were analyzed to determine the effects of the factors, treatment, harvested stage and mixture of oat, pea, and triticale on the measured variables. Simple statistics and the multivariate analysis of variance was performed. The seeding portions of oat, pea, and triticale denoted by X_1 through X_3, respectively. The dependent variables, i.e., the green mass yield (GM), dry matter yield (DM), yield of organic solids (OS), biogas yield from dry matter (BDM), biogas yield from organic solids (BOS), methane yield from dry matter (MDM), and methane yield from organic solids (MOS) are denoted by Y_1 through Y_7, respectively. The data of biogas yield (BDM and BOS) and methane yield (MDM and MOS) were, respectively, combined as Y_{4+5} and Y_{6+7} as there was no difference between them after the variance analyses (Table S1). The variables were analyzed using pairwise variables (X_1 and X_2, X_1 and X_3, X_2 and X_3) and quadratic and cubic polynomial regression models following [29–31]:

$$Y = \sum_{i=1}^{2} (\beta_{i\times j+1} X_i^j) + u \ (i = 1,\ 2;\ j = 1,\ 2) \tag{1}$$

$$(\text{i.e., } Y = aX_1{}^2 + bX_2{}^2 + dX_1X_2 + C;\ Y = aX_1{}^3 + bX_3{}^2 + dX_1X_3 + C;\ Y = aX_2{}^2 + bX_3{}^2 + dX_2X^3 + C) \tag{2}$$

where β is a constant.

Then, we obtained the preset condition linear model (Table S1) as:

$$100 = x1 + x2 + x3$$

and the linear models as:

$$Yi = ax1 + bx2 + cx3 \quad (i = 1,\ 2, \ldots\ 7) \tag{3}$$

where a, b and c are constants.

Then, the binary quadric fitting equations of the Ys were constructed as:

$$Yi = ax1^2 + bx2^2 + cx3^2 + dx1x2 + ex1x3 + fx2x3 + g \ (i = 1,\ 2, \ldots\ 7) \tag{4}$$

$$Yi = ax1^2 + bx2^2 + dx1x2 + g \quad (i = 1,\ 2, \ldots\ 7) \tag{5}$$

$$Yi = ax1^2 + cx3^2 + ex1x3 + g \quad (i = 1,\ 2, \ldots\ 7) \tag{6}$$

$$Yi = bx2^2 + cx3^2 + fx2x3 + g \quad (i = 1,\ 2, \ldots\ 7) \tag{7}$$

where a through g are constant. All of the models were solved.

The biologically meaningful models were selected for optimization. Response surface and contour charts are graphed for the meaning Ys with their corresponding X_s Figures 1 and 2. The biologically meaningful models were selected. Response surface and contour charts are graphed for Y_{4+5} through Y_{6+7} with their corresponding X_1 and X_3. The coefficients of the models are presented, and all the p values were significant at <0.05 in the models (Tables 2–4).

3. Results

3.1. Influence on Yield Traits, Biogas and Methane Yield

Extremely hot weather with a high amount of precipitation during the autumn period in both years was favorable for sowing and establishment of the winter crops until the end of October (Table 2). Large amounts of moisture during the winter and a rainy spring, especially in March and May in 2013, had a favorable impact on the growth and development of winter forage pea whose share increased linearly during the growing season in all mixtures with cereals. Large amounts of rainfall during 2014 in the first part of the growing season had a favorable effect on achieving high yields of winter pea as well of cereals. Cereals, especially oat, appeared to be much more aggressive due to intensive tillering, which was reflected in the lower share of pea in the mixtures (Table S2).

The analysis of variance illustrated that treatment, harvested stage, and their interactions had significant effects on green mass yield, dry matter yield, and yield of organic solids (Table 4), and on biogas yield and methane yield (Table 5). Additionally, the multivariate analysis of variance indicated that treatment and harvested stage significantly affected those indexes mentioned before (Table 6).

Table 4. Analysis of variance for green mass yield, dry matter yield and yield of organic solids for each experimental factor (treatment and harvested stage) and their interactions. Data included both years (n = 8).

Source (Factors)		Green Mass Yield		Dry Matter Yield		Yield of Organic Solids	
	DF	*F*-Value	*p*-Value	*F*-Value	*p*-Value	*F*-Value	*p*-Value
Treatment (T)	6	5.24	0.0004	40.64	<0.0001	43.62	<0.0001
Harvested stage (S)	1	39.03	<0.0001	86.66	<0.0001	60.01	<0.0001
T × S interaction	6	2.54	0.0344	3.70	0.0048	3.04	0.0146
Model	13	6.60	<0.0001	27.19	<0.0001	26.21	<0.0001
R-Square		0.6712		0.8936		0.8903	

Table 5. Analyses of variance for biogas and methane yield for each experimental factor (treatment and harvested stage) and their interactions. Data included both years (n = 8).

Source (Factors)		Biogas Yield		Methane Yield	
	DF	*F*-Value	*p*-Value	*F*-Value	*p*-Value
Treatment (T)	6	126.20	<0.0001	141.29	<0.0001
Harvested stage (S)	1	5.47	0.0213	29.44	<0.0001
T × S interaction	6	7.90	<0.0001	8.43	<0.0001
Model	13	62.31	<0.0001	71.34	<0.0001
R-Square		0.8921		0.9455	

Table 6. Multivariate Analysis of Variance (MANOVA) test criteria and F approximations for the hypothesis of no overall rffects of the factors. H = Type III SSCP matrix for the treatment and the harvested stage. NOTE: F Statistic for Roy's greatest root is an upper bound.

Factors	Statistic	Wilks' Lambda	Pillai's Trace	Hotelling–Lawley Trace	Roy's Greatest Root
Treatment (T)	*F*-value	12.37	5.70	22.50	108.02
	p-value	<0.0001	<0.0001	<0.0001	<0.0001
Harvested stage (S)	*F*-value	144.57	144.57	144.57	144.57
	p-value	<0.0001	<0.0001	<0.0001	<0.0001

Different treatments and harvested stages influenced green mass yield, dry matter yield and yield of organic solids (Figure 1). Treatment 4 (pure winter pea) had significantly lower green mass, dry matter and yield of organic solids compared to all other treatments, which had similar values for all three traits. The dry matter and organic solids for treatments 1, 2, 3, and 7 were significantly lower than for treatment 5.

Comparisons at different stages, the green mass yields for treatments 1 (pure oat), 2 (mixture of pea and out at the seed ratio 50%:50%), 6 (mixture of pea and triticale at the seed ratio 50%:50%) and 7 (mixture of pea and triticale at the seed ratio 75%:25%) were not significantly different between the two stages (Figure 1A), whereas for treatments 3 (mixture of pea and out at the seed ratio 75%:25%), 4 (pure pea) and 5 (pure triticale) significantly greater yields were observed at stage one (full-flowering stage of winter pea and beginning of milky stage of cereals) compared to stage two (emergence of first pods for pea and milky/waxy stage of cereals). For dry matter yield, treatments 3 and 4 had similar yields in both stages, whereas all other treatments (1, 2, 5, 6 and 7) had significantly greater dry matter yields at stage two of harvest compared to stage one (Figure 1B). The response of the yield of organic solids was like the response of dry matter yield, except for treatment 7, which had similar yields at both stages (Figure 1C).

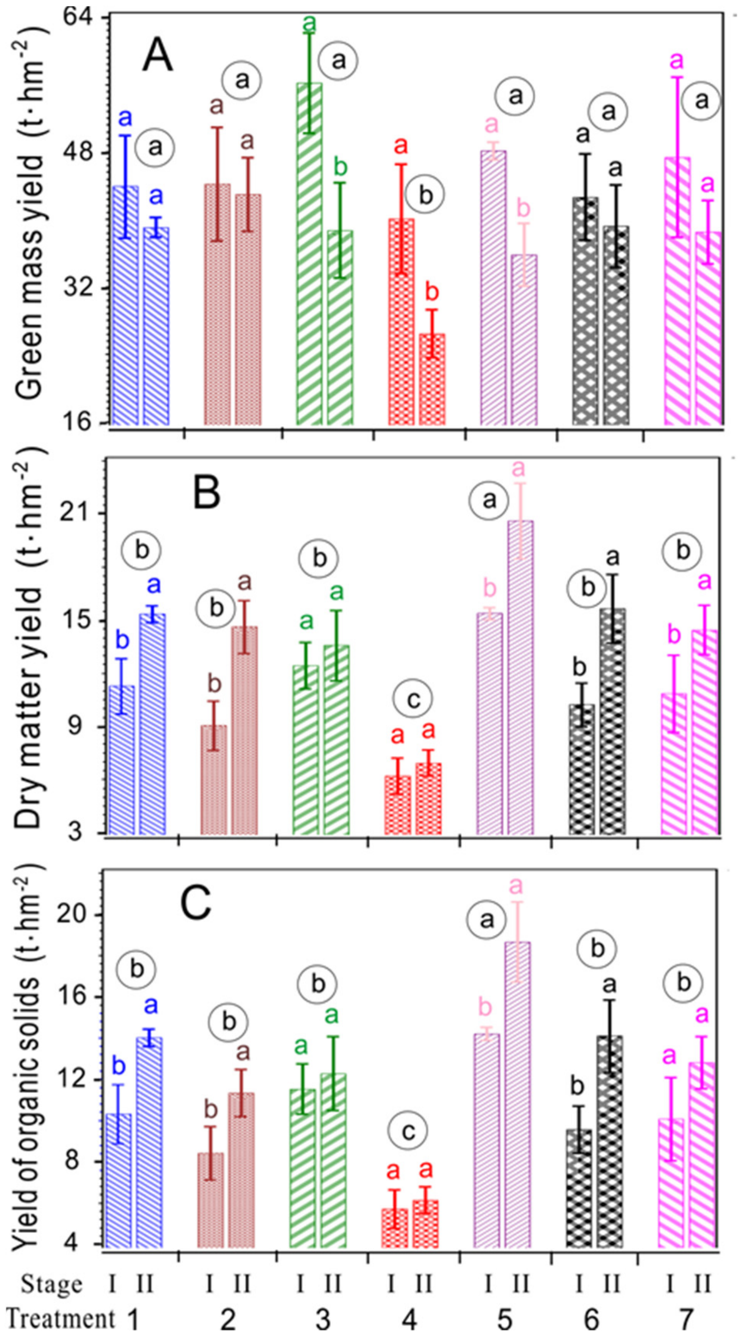

Figure 1. Yields of green mass (**A**), dry matter (**B**) and organic solids (**C**). The different lower letters in the circle indicated the difference among the treatments; the different colored lower letters indicate the difference between harvested stages I and II in their treatment. $n = 4$. Treatments include: 1: pure oat; 2: 50% winter pea:50% oat; 3: 75% winter pea:25% oat; 4: pure winter pea; 5: pure triticale; 6: 50% winter pea:50% triticale; 7: 75% winter pea:25% triticale. Stages include: 1. full-flowering stage of winter pea and beginning of milky stage of cereals and 2. emergence of firsts pods for pea and milky/waxy stage of cereals.

Biogas and methane yields were significantly influenced by treatments and stage of harvests (Figure 2A,B). Treatment 5 (pure triticale) produced the highest biogas and methane yields, whereas the lowest yields were observed in treatment 4 (pure winter pea) compared to all other treatments. While there were no significant differences in treatments 1 (pure oat), 3 (mixture of pea and out at the seed ratio 75%:25%), 6 (mixture of pea and triticale at the seed ratio 50%:50%) and 7 (mixture of pea and triticale at the seed ratio 75%:25%), they all had significantly greater biogas and methane yields than treatment 2 (mixture of pea and out at the seed ratio 50%:50%).

The biogas and methane at two stages of harvest across all treatment had similar responses in terms of yield. Significantly greater biogas and methane yields were observed at stage two (emergence of firsts pods for pea and milky/waxy stage of cereals) for treatments 1, 2 and 5. The harvest at stage one (full-flowering stage of winter pea and beginning of milky stage of cereals) was greater in treatments 4 and there was no significant difference between the two stages of harvest for treatments 3, 6 and 7.

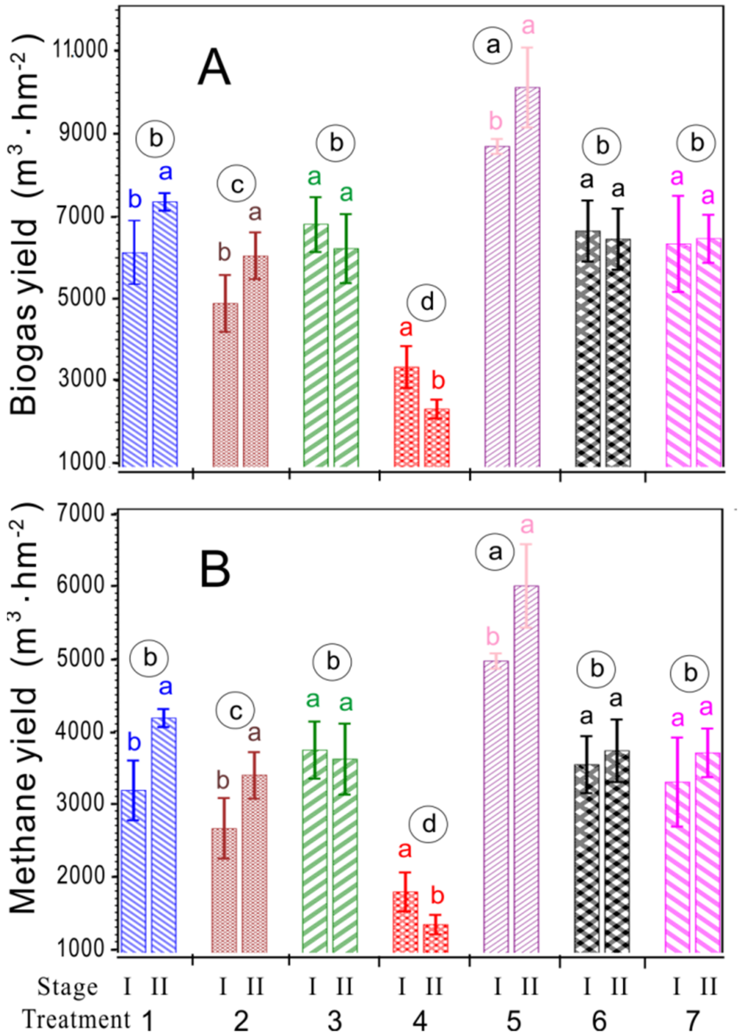

Figure 2. Yields of biogas (**A**) and methane (**B**). The different lower letters in the circle indicate the difference among the treatments; the different color lower letters indicated the difference between harvested stages I and II in their treatment. $n = 8$. Treatments include, 1: pure oat; 2: 50% winter pea:50% oat; 3: 75% winter pea:25% oat; 4: pure winter pea; 5: pure triticale; 6: 50% winter pea:50% triticale; 7: 75% winter pea:25% triticale. Stages include: 1. full-flowering stage of winter pea and beginning of milky stage of cereals and 2. emergence of firsts pods for pea and milky/waxy stage of cereals.

3.2. Surface Plots to Determine Optimal Mixtures for Biogas and Methane Yields

To determine the optimum mixture of oat and triticale, a surface plot model was developed which showed the significant impact of different combinations on biogas and methane yields (Table 7).

Table 7. Analyses of variance for the models of the biogas, methane, green mass and organic solids yields, for experimental factor oat (X_1) and triticale (X_3). Data included for both years (n = 8).

Source (Factors)		Biogas Yield		Methane Yield		Green Mass Yield		Organic Solids	
	DF	*F*-Value	*p*-Value	*F*-Value	*p*-Value	*F*-Value	*p*-Value	*F*-Value	*p*-Value
Oat	2	37.02	<0.0001	31.56	<0.0001	11.69	<0.0001	33.11	<0.0001
Triticale	2	115.14	<0.0001	118.02	<0.0001	0.17	0.8458	29.43	<0.0001
Model	4	58.95	<0.0001	60.06	<0.0001	12.74	<0.0001	20.51	<0.0001
R-Square		0.6879		0.6919		0.3226		0.4340	
Maximum		13218		8595		61.48		25.64	

Response surface graphs were plotted with pair-wise variables for biogas and methane yields for combinations of oat and triticale (Figure 3). The results showed that there were maximum stable points for both biogas and methane yields. The maximum biogas yield of 10,252 m^3 ha^{-1} was obtained when the mixture of oat and triticale, respectively, was at 35% and 65% (Figure 3A). Similarly, the maximum methane yield of 6625 m^3 ha^{-1} was obtained when the mixture of oat and triticale, respectively, was at 27% and 73% (Figure 3B).

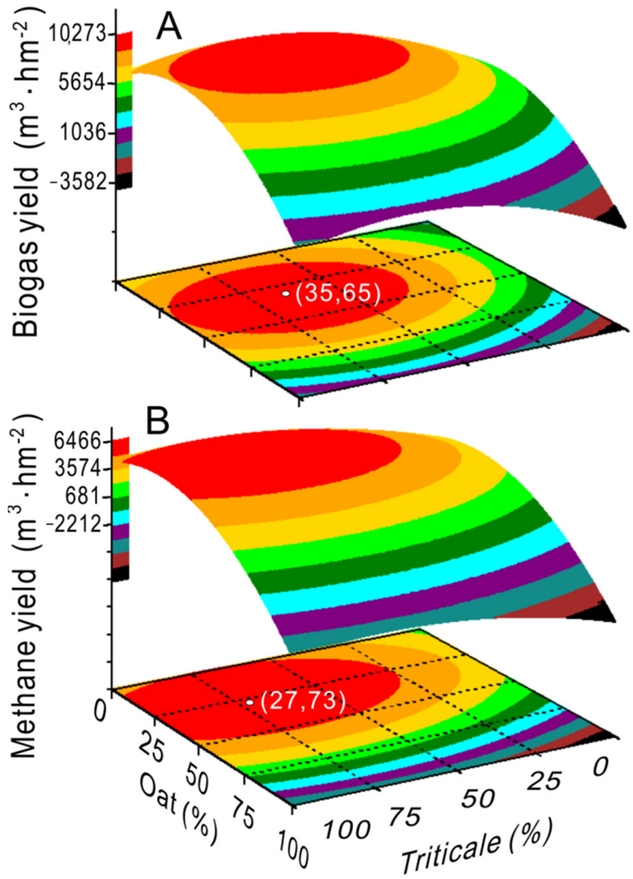

Figure 3. Response surface plots showing the interactions between oat and triticale on the biogas yield (**A**) and methane yield (**B**).

After quadratic model analyses, the combinations ratios of the oat and triticale were, respectively, at 30% with 8% and 28% with 38%, whereas the green biomass and organic solid yields had maximums of 61.48 and 25.64 t ha^{-1}, respectively (Figure 4). When the model has preset the three independent variables oat, pea and triticale, the sum of them is 100%, pea should be 62% (100-30-8) and 34% (100-28-38), respectively, in the optimal productive combination of green mass and organic solids yields. The factor triticale was not significant (p = 0.8458) in terms of the variable green mass yield, but the model was significant (Table 7). However, there were not any biological suitable models either with the combination of oat and pea or triticale and pea due to not significant models or saddle point with a minus value of the factor.

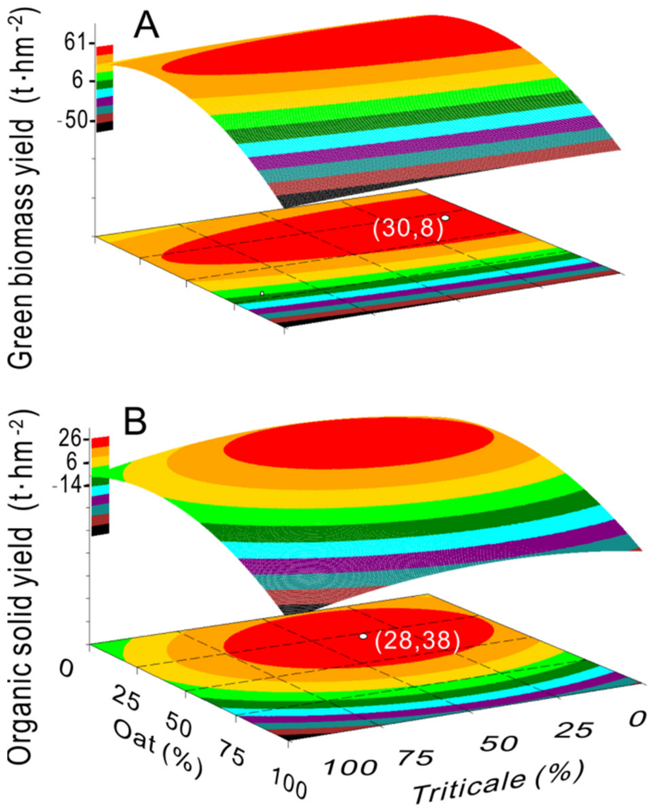

Figure 4. Response surface plots showing the interactions between oat and triticale on the green biomass yield (**A**) and organic solid yield (**B**).

4. Discussion

There were significant impacts of different crop mixtures (pea and cereals) and stage of harvest on biomass production and yields of biogas and methane. Many conventional forage crops are easy to cultivate and produce large amounts of biomass. Several studies that analyze and compare the methane production potential of different crop species already exist [32,33]. Different parameters have been reported to be correlated with the methane production potential of biomasses and several models have been developed for prediction of specific methane yields from biomasses (Table 8). The most important parameter in choosing crops for methane production is the net energy yield per hectare, which is defined mainly by biomass yield and convertibility of the biomass to methane, as well as cultivation inputs. Maximum green biomass produced from the mixture of pea and oat was at the seed ratio 75%:25% when compared to all other treatments and mixtures (Figure 1). The green mass was also greater at the first stage across all treatments.

Table 8. Coefficients of the correlation with the dependent variables. Note: GM: the green mass yield; DM: dry matter yield; OS: yield of organic solids; BDM: biogas yield from dry matter; BOS: biogas yield form organic solids; MDM: methane yield from dry matter; MOS: methane yield from organic solids. Significance of the correlation were at of **: $p < 0.001$; ***: $p < 0.0001$.

	DM	OS	BDM	BOS	MDM	MOS
GM	0.223	0.248	0.448 **	0.446 **	0.372 **	0.368 **
DM	1.000	0.988 ***	0.899 ***	0.899 ***	0.932 ***	0.929 ***
OS		1.000	0.929 ***	0.929 ***	0.957 ***	0.956 ***
BDM			1.000	0.999 ***	0.992 ***	0.991 ***
BOS				1.000	0.992 ***	0.990 ***
MDM					1.000	0.998 ***

Our results showed that yield of dry matter and organic solids, harvested at the first maturity stage, is significantly higher than at the second stage (Figure 1). The impacts of harvesting time may be due to the fact that capacity of nitrogen supply in soil and the nitrogen absorption efficiency of plants changed, which were crucial to carbon and nitrogen metabolism, with different growth stages, thereby affecting the yield and quality of plants [34]. A similar trend was seen in the yields calculated in other studies conducted on rye (*Secale cereale* L.), where the latest harvest date at late milk ripening resulted in the highest dry matter yield on a whole plant level with an average of 16.0 t ha^{-1}. Accordingly, methane yield reached a mean of 4424 m^3 ha^{-1} and a maximum of 4812 m^3 ha^{-1} [35]. The production of organic matter and nutritional compositions and structures were essential to biogas and methane yield [36]. Six different varieties of silage maize had the highest methane yield during late harvest (6270 m^3 CH_4 (104 $m^2)^{-1}$) [37].

Switchgrass showed the opposite trend with to above literature; special methane yields decreased significantly with crop maturity, from 0.266 to 0.309 normalized liter per gram of volatile solids of energy crop [$_N$L CH_4 (g VS)$^{-1}$] to 0.191–0.250 NL CH_4 g^{-1} vs. [38]. Similarly, the harvest time significantly affected methane yield of switchgrass grassland biomass. With the delay of harvest time, the methane yield decreased significantly, from up to 309 ln kg^{-1} organic dry matter (ODM) in May to below 60 ln kg^{-1} ODM in February [39]. The reason for this phenomenon is related to the increase in lignin content [40]. Lignocellulosic hydrolysis is generally considered as a rate-limiting step in anaerobic digestion [41–43]. Therefore, energy crops need to be pretreated with the purpose of separating and removing lignin from biomass, increasing the porosity of biomass and the conversion rate of enzymes to cellulose, so as to improve the conversion rate of hemicellulose and cellulose in biomass, and finally effectively improve the gas production performance of raw materials [44,45].

In our research, the highest and lowest values of DM and OS were observed in pure crops: in treatment 5 (pure triticale) and treatment 4 (pure winter pea), respectively (Figure 1). There were no differences among other treatments. A similar trend was observed, with the highest and lowest biomasses in treatments 5 and 4, while treatment 3 (mixture of pea and oat at the seed ratio 75%:25%) had slightly lower yields than all other treatments, but more than treatment 4. The response of treatment for biomass and methane followed the same trend, suggesting strong correlations between the two products and their relations with the total dry matter production and yield of organic solids. Biogas generated through anaerobic digestion included about 60% methane [46]. However, GM saw different patterns with DM, OS, methane, and biogas yields; the highest and lowest values were in treatments 3 and 4, respectively (Figure 1). This may be because during drying (which is equal to the procedure of late ripening), nutrients were redistributed from source to sink [47].

Generally, later harvest stages had greater biomass and methane yields with an exception of treatment 4 (Figure 2). In addition, pure triticale had the highest DM, organic solids, and yields of methane and biogas at later stages of crop development (Figures 1 and 2). Research showed that as plants mature, there is increasing structural plant fibrillation and lignification and the accumulation of highly digestible carbohydrates [48]. Research has suggested that triticale was not only resistant, but also combined the advantages of rye and wheat, with the ability to use nutrition effectively and a high grain yield and nutritional quality [49]. In contrast, all the index values in pure winter pea (treatment 4) were the lowest, suggesting a relatively poor performance. This could be explained by the result that pea's yield component, biotic and abiotic stress resistance, seed quality, stability of yield were not good enough [50].

The result of modeling analyses, the highest biogas yield of 10,252 m^3 hm^{-2} produced by the mixture of oat (35%) with triticale (65%) (Figure 3A) was more than 0.8 m^3 hm^{-2} produced by pure triticale at stage II (Figure 2A), whereas the highest methane yield of 6625 m^3 hm^{-2} produced by the mixture of oat (27%) with triticale (73%) was more than that of 6007 m^3 hm^{-2} by the same treatment (Figure 2B). The results showed that co-digestion of oat and triticale generated more biogas and methane yields than digestion of sole triticale. Similar findings were also reported from other studies [51,52]. This probably is because in the process of co-digestion, some beneficial parameters describing good digester health performed better, such as pH, alkalinity, and carbon–nitrogen ratio [53].

There are also some studies about improving biogas and methane production of previous materials. The biogas production ranges from 438 to 852 Nm(3)/t of dry matter for wheat and ear maize silage, respectively [54]. CH_4 yield (MY; mg of CH_4/g DM degraded) of barley and oat grown at one location of incubation was less than that of wheat and triticale (28 vs. 31 mg CH_4/g DM degraded) [55]. The optimum addition was a 1:2 ratio of cow manure to the oat straw added, which resulted in a suitable C/N ratio of 27 and a higher degradation rate of lignocellulose, and this condition had the best cumulative methane yield of 841.77 mL/g volatile solids added (VS_{added}), 26.64% greater than that of digesting oat straw alone [56]. The methane yield for oat was 841.77 mL/g volatile solids added [57]. Since most of the results mentioned above were based on laboratory levels rather than field levels in our experiments, there is no uniform standard to compare them to, which is worth studying.

Additionally, there were other important factors in the process of methane and biogas production, for example, substrates [13,58], enzymes [59–62], microorganisms [13,63,64], and fermentation conditions [65,66]. Further studies are needed in the future about relevant mechanisms (Zhang et al., 2013) which could be achieved by physiological, biochemical, molecular [67,68], and omics analyses [69,70]. More importantly, the ecological use of energy crops to produce biogas needs to be further developed, such as exploiting crops accumulating heavy metal produced biogas [14]. Finally, with the development of energy crops, anaerobic digestion costs will increase and there will be contradictions between foods and feeds as well. Solutions to this problem such as considering resources such as agricultural wastes as alternatives or supplements of energy crops are to be further explored [71,72]. It is possible that the seeding ratio can also have direct influence on biomass ratio, which in turn can impact biogas and methane yields. Further research on this topic is needed to optimize the system.

5. Conclusions

The used pure or mixtures of pea, oat and triticale produced different green mass, dry matter, solids, biogas and methane yield. The experiments showed that maximum green mass was produced by the mixture of pea and oat at the seed ratio 75%:25% and when crops were harvested at the first stage of maturity (full-flowering stage of winter pea and beginning of milky stage of cereals). The pure triticale produced biogas and methane yields similar to other cropping mixtures. A multivariate model was developed to identify the best combinations for biogas and methane yields. These results showed that the mixture of oat and triticale had great potentiality with regard to biogas and methane yields. The optimal mixture was oat (35%) with triticale (65%) for biogas yield and oat (27%) with triticale (73%) for methane yield. After quadratic model analyses, the combinations ratios of the oat and triticale were, respectively, 30% with 8% and 28% with 38%, whereas the green biomass and organic solid yields were at maximums of 61.48 and 25.64 t ha^{-1}, respectively. When the model preset the three independent variables oat, pea and triticale (sum as 100%), the proportion of pea was 62% and 34% for optimum production of green mass and organic solid yields, respectively. If these crop mixtures are to be used for biofuel production or forage, an appropriate cropping mixture should be selected. However, these need to be further evaluated under different environmental and soil conditions, and a cost–benefit ratio should be determined.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091800/s1, Figure S1. Precipitation and mean monthly temperature for growing season (2012–2014) and long-term average (1963–2013); Table S1. The portions of the three factors oat ($X1$), pea ($X2$) and triticale ($X3$) transformed at 0.95 confidence interval from the original experimental design; Table S2. Share of pea (P-%) and cereals (oats-O-% and triticale-T-%) in the two different maturity stages in period 2013–2014.

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