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Establishment and production of lucerne (*Medicago sativa* L.) in Sweden is affected by inoculation product choice

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ABSTRACT

Lucerne (*Medicago sativa* L.) is an important perennial forage legume in Sweden, but its potential cultivation area is constrained by uncertainty of successful establishment. This study aimed to identify management practices that could lead to improved establishment of lucerne. Lucerne cultivar SW Nexus was grown at four different locations in southern Sweden over two establishment/production cycles. At all locations except Svalöv, lucerne had not previously been cultivated in the plot location for at least seven years. Inoculation treatments of one standard rhizobia (Nitragin Gold, NG), two NG-related, three NGs combined with single micronutrient, and six alternative inoculants were assessed in comparison with a no inoculation control for their effects on lucerne establishment and production. The results showed that alternative inoculants were sometimes better than the standard inoculants. The largest contrast between different inoculation treatments was at Rådde in the first crop cycle, where the best treatment yielded 12000 kg DM ha^{-1} across three harvests, nearly twice that of the control, and all alternative inoculant treatments had higher total nitrogen concentration (TN), lower carbon to nitrogen ratio (C:N), and greater normalised difference vegetation index (NDVI) than the control. There was no evidence that the soil-applied micronutrients improved yield at any location. At Svalöv, where lucerne had previously been grown, there was no effect of any of the treatments. In conclusion, inoculation is essential at locations where there is no history of lucerne cultivation, and choice of inoculation product can affect establishment and production.

1. Introduction

Lucerne (*Medicago sativa* L.) is the most widely grown (over 32 million hectares worldwide) forage legume in the world [1]. Because of its high nutritional content and high digestibility, lucerne has long been recognised as an important forage crop for ruminant feeding [2,3]. At high latitudes in Europe, forage is the most important crop and dominates agricultural land use [4]. Due to its persistence and cold tolerance, lucerne is a feasible alternative or complement to red clover in Sweden. Lucerne is also drought tolerant due to its deep root system, which is an important characteristic as the climate becomes potentially more variable and drier [5]. Increased use of lucerne could reduce the risk of ley production and provide high-quality feed for ruminant producers. However, its potential cultivation area is constrained by issues with establishment [6].

Lucerne is sensitive to the environment; soils with suitable pH (6.0–8.5), adequate drainage, sufficient macro and micronutrients, and specific rhizobia are its specific environmental requirements for growth [7,8]. Failure to provide these conditions can result in establishment failure, which highlights the importance of site selection, nutrient application and rhizobia inoculation. Therefore, choosing appropriate management practices is important for establishment of lucerne.

Seed inoculation with species-specific rhizobia (mainly *Sinorhizobium meliloti*) to enable nodulation is essential for soils without previous lucerne history [9]. The presence of sufficient micronutrients (particularly B, Mo and Fe, but also Cu, Mn, Zn, and Co) is another condition of successful establishment [10]. However, there are few studies available on the effects of inoculation and micronutrients on

Abbreviations: B, Boron; Mo, molybdenum; Fe, iron; Cu, copper; Mn, manganese; Zn, zinc; Co, cobalt; RCBD, randomised complete block design; NG, Nitragin Gold; DM, dry matter; N, nitrogen; C, carbon; TN, total nitrogen; TC, total carbon; NDVI, normalised difference vegetation index; NIR, near-infrared.

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lucerne establishment and production at high latitudes [11]. In this study, 12 inoculation treatments (11 in the first year) were applied to lucerne before sowing at four sites in south Sweden for two establishment/production cycles. We aimed to identify management practices that could lead to improved lucerne establishment. The hypotheses of this study were (i) choice of inoculation products/techniques can result in differences in yield and nitrogen (N) concentration of lucerne, (ii) application of micronutrients can increase yield and N concentration of lucerne where soil test results indicate deficiencies in micronutrients, (iii) inoculation effects can be large in soils without a history of lucerne cultivation.

2. Materials and methods

2.1. Site description and soil characteristics

The field experiments were conducted at four sites in southern Sweden: Svalöv (55°55'20.06"N, 13°7'32.24"E), Rådde (57°36'31.73"N, 13°15'43.35"E), Tenhult (57°44'0.87"N, 14°17'10.31"E) and Lilla Böslid (56°35'50.5"N, 12°57'5.2"E), over two establishment and production cycles (2019/2020, referred to as experiment 1 and 2020/2021, referred to as experiment 2). At Rådde, Tenhult and Lilla Böslid, lucerne had not previously been cultivated in the plot locations for at least seven years. Svalöv was a control site, where lucerne was known to have been grown recently.

The annual average rainfall of Svalöv, Rådde, Tenhult and Lilla Böslid are 671, 1057, 587 and 714 mm, respectively, while the monthly average temperatures range from 1.2 to 19.9, −2.1 to 17.5, −2.4 to 16.9 and 0.7 to 7.9 °C, respectively. Average air temperature and precipitation during the growing period throughout the experiment (Fig. S1) were obtained from an online gridded database available at the Swedish Meteorological and Hydrological Institute. The soil at each site was classified as loamy sand, which is an appropriate soil type for cultivating lucerne. Before sowing, the surface layer soil (0–15 cm) at each site was sampled and sent to Eurofins Agro Testing (Kristianstad, Sweden) for analysis of physiochemical properties. Available Mo, Co and B concentrations were analysed using Inductively coupled plasma-mass spectrometry (ICP-MS) (Table 1).

2.2. Plant material and culture

The Swedish lucerne cultivar SW Nexus (Lantmännen, Svalöv, Sweden) was used in this study. It is the Swedish lucerne reference cultivar for variety trials and is commonly used in the Nordic countries.

2.3. Experimental design and treatments

The experiments had a randomised complete block design (RCBD) with three replicates in 2019/2020 and four replicates in 2020/2021 (three at Svalöv). For both experiments, the sowing plot sizes were 9.60 × 1.13 m at Svalöv, 12.0 × 1.75 m at Rådde, 15.4 × 1.75 m at Tenhult and 12 × 1.5 m at Lilla Böslid. The adjacent plots were separated by a buffer zone (0.5 m). After field preparation, basal fertilisers were applied according to soil tests and standard recommendations (Table S1). Experiment 1 at Lilla Böslid was discarded due to high weed pressure that could not sufficiently be controlled. Experiment 2 at Tenhult was discarded due to the negative effects of a residual herbicide sprayed on the preceding crop.

The experimental treatments included one control (T1), one standard inoculant treatment (Nitragin Gold, NG, T2), two NG-related inoculant treatments (T3–T4), three NG combined with single micronutrient treatments (T5–T7) and six alternative (i.e. not NG) inoculant treatments (T8–T13) (five in experiment 1). For T5, the application rate of B was 22.0 kg borate ha^{-1} (2.50 kg B ha^{-1}). Borate was mixed with fine sand to 'bulk it up' and applied by hand. For T6 and T7, the application rates were 252 g sodium molybdate (Na_2MoO_4) ha^{-1} (100 g Mo ha^{-1}) and 477 g cobalt sulphate ($CoSO_4$) heptahydrate ha^{-1} (100 g Co ha^{-1}), respectively. Both Mo and Co were supplied as concentrated solutions using watering cans; the concentrations of the supplied solution were 0.280 g Na_2MoO_4 ml^{-1} and 0.302 g $CoSO_4$ ml^{-1}, respectively. ThermoSeed® is a seed treatment with steam that pasteurizes seeds to reduce seed-borne diseases. The detailed experimental design and treatments are listed in Table 2.

Lucerne seeds were sown as a monoculture (without a cover crop) on 27, 19 and 25 June 2019 at Svalöv, Rådde and Tenhult for experiment 1, and on 3 June, 6 May and 11 June 2020 at Svalöv, Rådde and Lilla Böslid for experiment 2, with ten rows per plot (nine at Svalöv). Inoculants for individual treatment were prepared according to manufacturers'

Table 2
Details of individual treatments in the experiments.

No.	Treatments	Details
T1	Control	No inoculant or nutrient treatment
T2	Nitragin Gold (NG)	Novozymes A/S, Bagsvaerd, Denmark
T3	NG 5 × rate	Novozymes A/S, Bagsvaerd, Denmark
T4	NG + ThermoSeed®	Lantmännen BioAgri, Uppsala, Sweden
T5	NG + Molybdenum (Mo)	Novozymes A/S, Bagsvaerd, Denmark
T6	NG + Cobalt (Co)	Novozymes A/S, Bagsvaerd, Denmark
T7	NG + Boron (B)	Novozymes A/S, Bagsvaerd, Denmark
T8	SAS Gold	Jouffray-Drillaud, Cisse, France
T9	SAS GR01	Jouffray-Drillaud, Cisse, France
T10	SAS Life	Jouffray-Drillaud, Cisse, France
T11	Pellifix	Legume Technology, East Bridgford, UK
T12	LegumeFix + Lime coating	Legume Technology, East Bridgford, UK
T13	Prolime 100[a]	Prolime AG, Laingsburg, USA

[a] Only in experiment 2.

Table 1
Basic physicochemical properties of the soils in the experiments[a].

	Experiment 1			Experiment 2		
Properties	Svalöv	Rådde	Tenhult	Svalöv	Rådde	Lilla Böslid
Soil texture (sand: silt: clay)	75:8:13	79:12:2	84:8:6	68:15:14	62:28:4	66:12:15
Organic matter (%)	3.7	7.0	2.4	2.8	5.4	6.3
pH (water extraction)	6.3	5.1	5.5	6.3	5.6	5.8
CEC (mmol^{+} kg^{-1})	123	51	42	131	57	132
Total N (kg ha^{-1})	3060	3750	1580	4420	15600	7500
Available P (kg ha^{-1})	3.2	0.3	4.1	5.2	2.4	1.8
Available K (kg ha^{-1})	50	65	75	160	305	185
Available S (kg ha^{-1})	26	12	11	26	51	26
Available Ca (kg ha^{-1})	185	15	120	30	650	245
Available Mg (kg ha^{-1})	55	60	100	150	305	345
Available Mo (kg ha^{-1})	<10	<10	<10	<10	<30	<10
Available Co (kg ha^{-1})	5	35	10	<10	25	<10
Available B (kg ha^{-1})	445	265	15	895	855	2240

[a] Soil analysis methods: Soil texture, organic matter, pH, Total N, CEC, Available Ca, Mg, Mo, Co, B – Em:NIRS(TSC®); Available P, K, S, Mg – Em:CCL3(PAE®).

instructions. Plots were not irrigated, as is normal practice for forages in Sweden. Information on fertiliser application is shown in Table S2. In experiment 1, at Svalöv, lucerne was cut on 29 August 2019 to control weeds. During the establishment year at each site, weeds were either removed by hand, sprayed with herbicide or removed with a forage harvester.

2.4. Measurement

2.4.1. Dry matter yield

Lucerne was harvested multiple times with a stubble height of approximately 8 cm, one time in the establishment years and three times in the production years (Table 3). Hereafter, these harvests are referred to as "establishment harvest", "harvest 1", "harvest 2", and "harvest 3". Lucerne at Svalöv was not harvested in the first establishment year (2019), as biomass was lower due to an earlier cut to control annual weeds. The harvest plot sizes were 8.80, 10.4, 11.0, and 12.5 m^2 at Svalöv, Rådde, Tenhult and Lilla Böslid, respectively. Biomass samples were collected from each plot and dried at 105 °C until a constant weight was reached, to determine the dry matter (DM) content and calculate the DM yield of each harvest. Additional samples were dried at 60 °C and stored for further nutrient analysis.

2.4.2. Nitrogen (N) and carbon (C)

Samples from the establishment year and the first harvest in the production year were used for analysis of N and C. Shoot samples were milled to 1-mm particle size, and the total nitrogen and carbon concentrations (TN and TC) were determined using an elemental analyser (TurMac, LECO, Saint Joseph, USA). The C:N ratio was calculated by the formula:

$$\text{C : N ratio} = \frac{\text{C content } (\text{mg kg}^{-1})}{\text{N content } (\text{mg kg}^{-1})} \quad (1)$$

2.4.3. Normalised difference vegetation index (NDVI)

Our previous study showed that NDVI has excellent potential for separating inoculation treatments in fields [12]. A GreenSeeker handheld active light crop sensor (Trimble, Sunnyvale, USA) was used for NDVI measurement shortly before each harvest. The measurement was taken from the beginning to the end of each plot at approximately 1 m above the ground. NDVI is defined as follows:

$$\text{NDVI} = \frac{\rho_{nir-}\rho_{red}}{\rho_{nir+}\rho_{red}} \quad (2)$$

where ρ_{nir} and ρ_{red} are reflectance of near-infrared and visible red, respectively.

2.5. Statistical analyses

Treatment results are means of three (all sites in experiment 1 and Svalöv in experiment 2) or four (all sites in experiment 2 except Svalöv) replicates. The statistical analyses were conducted separately for each site using Proc Glimmix in SAS (version 9.4, SAS Institute Inc., Cray, USA). The model was constructed using block, treatment, harvest, and treatment × harvest interaction as fixed effects. To accommodate for repeated measurements taken from the same plot, harvest was nested within the plot as a random effect. To analyse the effects on combined dry matter yields of all harvests, the model was constructed using block and treatment as fixed effects. Differences among treatments were determined using Tukey's test at a significance level of $P < 0.05$.

3. Results

3.1. Yield

In the first establishment/production cycle (2019/2020), DM yields at Rådde and Tenhult were significantly affected by treatments (Table 4). In the second establishment/production cycle (2020/2021), treatment only significantly affected DM yield at Rådde. Dry matter yield significantly differed between harvests for all sites and years. There was an interactive effect of treatment and harvest on DM yield in experiment 1 at Tenhult and Rådde. The interactions are depicted in Table 4.

At Rådde, in the first establishment year (2019), four alternative inoculant treatments had significantly higher yields than control, and the two best alternative inoculant treatments (Clay Pellifix and LegumeFix + lime coating) yielded significantly higher than NG (Fig. 1). For three harvests in the production year and the total yield, all inoculation treatments yielded significantly higher than control; alternative inoculant treatments yielded higher than NG and NG-related micronutrient treatments. The combined yield of the best treatments (SAS GR01 and LegumeFix + lime coating) reached approximately 15000 kg DM ha^{-1}, significantly greater than control (Fig. 1).

At Tenhult, for the establishment harvest and for harvest 1 of the production year, seven inoculation treatments had significantly higher yields than control, but none were significantly different from each other (Fig. 1). For production year harvest 2, seven inoculation treatments yielded higher than control, and the best inoculant (LegumeFix + lime coating) yielded significantly higher than the worst one (NG + Co). No significant differences were found for production year harvest 3. Across all harvests, none of the 11 inoculation treatments were significantly different to each other; however, all but one inoculant (NG, Thermoseed) were more productive than the control.

At Svalöv, lucerne was not harvested in the establishment year, as biomass was lower due to an earlier cut to remove weeds. However, Svalöv had the highest total yields among all sites. There were no significant differences between treatments for any harvest in the production year (Fig. 1).

At Lilla Böslid, there were minor differences between treatments; the results are not shown due to the influence of weeds at this site in 2019/2020, and subsequent poor quality of the data.

In the second establishment/production cycle (2020/2021), the differences among treatments at all sites were less than for the first experiment (Fig. 2). At Rådde, in the second establishment year, only one alternative inoculant treatment (SAS Life) yielded higher than control, and there were no significant differences among other treatments. No significant differences were observed for any harvest in the production year. At Svalöv and Lilla Böslid, there were no differences in yield among treatments for any harvest. The 2020/2021 experiment at Tenhult was discarded due to poor establishment resulting from the residual effect of a herbicide.

Table 3
Harvest dates in the experiments. E: establishment year. H: harvests.

Harvests	Experiment 1			Experiment 2		
	Svalöv	Rådde	Tenhult	Svalöv	Rådde	Lilla Böslid
E	–	1 October 2019	25 September 2019	23 September 2020	17 September 2020	27 August 2020
H1	16 June 2020	17 June 2020	24 June 2020	9 June 2021	23 June 2021	18 June 2021
H2	3 August 2020	21 July 2020	6 August 2020	21 July 2021	23 July 2021	27 July 2021
H3	23 September 2020	23 September 2020	28 September 2020	8 September 2021	8 September 2021	22 September 2021

Table 4
Type III tests of fixed effects for dry matter yield, total nitrogen (TN), and C:N ratio in response to different treatments (Trt) and harvests (H).

	Experiment 1 (2019/2020)									Experiment 2 (2020/2021)								
	Svalöv			Rådde			Tenhult			Svalöv			Rådde			Lilla Böslid		
	DF	*F*	*P*	DF	*F*	*P*	DF	*F*	*P*	DF	*F*	*P*	DF	*F*	*P*	DF	*F*	*P*
Yield																		
Trt	11	0.54	0.871	11	18.01	<0.001	11	5.99	<0.001	12	1.29	0.605	12	2.60	0.007	12	1.22	0.273
H	2	1335	<0.001	3	626	<0.001	3	927	<0.001	3	718	0.027	3	1516	<0.001	3	631	<0.001
Trt × H	22	0.62	0.893	33	2.17	0.007	33	2.60	0.002	36	0.79	0.731	36	0.87	0.679	36	0.82	0.749
TN																		
Trt	/	/	/	11	22.6	<0.001	11	3.36	0.007	/	/	/	12	0.33	0.979	12	1.53	0.175
H	/	/	/	1	74.7	<0.001	1	741	<0.001	/	/	/	1	0.42	0.520	1	410	<0.001
Trt × H	/	/	/	11	1.86	0.098	11	1.59	0.167	/	/	/	12	1.16	0.343	12	0.69	0.754
C:N																		
Trt	/	/	/	11	20.5	<0.001	11	2.75	00.019	/	/	/	12	0.38	0.963	12	1.5	0.179
H	/	/	/	1	117	<0.001	1	2443	<0.001	/	/	/	1	0.41	0.525	1	301	<0.001
Trt × H	/	/	/	11	3.68	<0.001	11	3.04	0.011	/	/	/	12	1.21	0.313	12	1.07	0.409
NDVI																		
Trt	11	0.41	0.928	11	8.36	<0.001	11	2.40	0.045	12	0.56	0.846	12	0.69	0.743	12	0.86	0.589
H	3	461	<0.001	3	1106	<0.001	3	128	<0.001	3	78.3	<0.001	3	529	<0.001	3	43.7	<0.001
Trt × H	33	1.32	0.209	33	12.1	<0.001	33	1.47	0.133	36	0.95	0.566	36	0.91	0.610	36	1.21	0.243

12 treatments in experiment 1 and 13 treatments in experiment 2. All measurements were done shortly before harvesting; one measurement was done in each establishment year and 3 measurements were done in each production year.

3.2. Nitrogen and carbon analyses

3.2.1. Total nitrogen

In the first establishment/production cycle (2019/2020), TN at Rådde and Tenhult was significantly affected by treatments (Table 4). There were no significant treatment effects in experiment 2 (2020/2021) at Rådde and Lilla Böslid. Total nitrogen significantly differed between harvests at Rådde and Tenhult in experiment 1, and at Lilla Böslid in experiment 2. No significant interactions were observed between treatments and harvests.

In experiment 1 (2019/2020), the differences were more evident in the establishment year (2019) than in the production year (2020) both at Rådde and Tenhult. In the establishment year (2019), at Rådde, all alternative inoculant treatments but no other inoculation treatments had higher TN than the control (Table 5). At Tenhult, eight inoculation treatments had significantly greater TN than the control, but none of them were significantly different from each other. There were no significant differences among treatments at Svalöv in the production year (2020). For the second experiment, there were no significant differences in N concentration between any treatments. Averages of control and treatments are presented in Table 6.

3.2.2. Carbon to nitrogen ratio (C:N)

Treatment significantly affected C:N ratio at Rådde and Tenhult in experiment 1 (2019/2020) but not in experiment 2 (2020/2021) (Table 4). Harvest time significantly affected C:N at Rådde and Tenhult in experiment 1, and at Lilla Böslid in experiment 2. Interactions of treatment and harvest time on C:N ratio at Tenhult and Rådde were significant in experiment 1 (2019/2020) but not in experiment 2 (2020/2021). At Tenhult, C:N ratios for all treatments in the production year were greater than the establishment year, whereas at Rådde they were lower in the production year when compared with the establishment year (Table 7).

In experiment 1, at Rådde, alternative inoculant treatments had significantly lower C:N ratio than control and some NG-related micronutrient treatments in both the establishment and production year (Table 7). At Tenhult, all inoculation treatments had significantly lower C:N ratio than control in the establishment year (2019). Three alternative inoculant treatments (SAS Gold, SAS GR01 and LegumeFix + lime coating) had significantly lower C:N ratio than control at the first harvest in the production year, but there were no significant differences among any inoculation treatments.

3.3. NDVI

In the first establishment/production cycle, NDVI at Rådde and Tenhult were significantly affected by treatment, whereas there were no significant effects of treatment on NDVI in the second establishment/production cycle (2020/2021) (Table 4). There was no significant treatment effect on NDVI at Svalöv in any year. Harvest time significantly affected NDVI at all sites in all years (Table 4). There was an interaction of treatment and harvest time in experiment 1 (2020/2021) at Rådde (Table 4).

At Rådde, NDVI in three treatments was significantly greater than control at the establishment harvest, and production year harvests 1 and 2 of experiment 1 (2019/2020) (Table 8). All of the five alternative inoculant treatments had greater NDVI than others in the establishment year, while four alternative inoculant treatments had significantly greater NDVI than the three NG-related micronutrient treatments at harvest 1 in the production year. At harvest 3, all but two inoculation treatments had NDVI greater than control. None of the treatments were significantly different from each other in production year harvests 2 and 3. The NDVI of NG and NG-related inoculants and micronutrient treatments increased over time. For alternative inoculant treatments, there was no general pattern in NDVI. Alternative inoculant treatments had higher NDVI than all other inoculation treatments at all harvests.

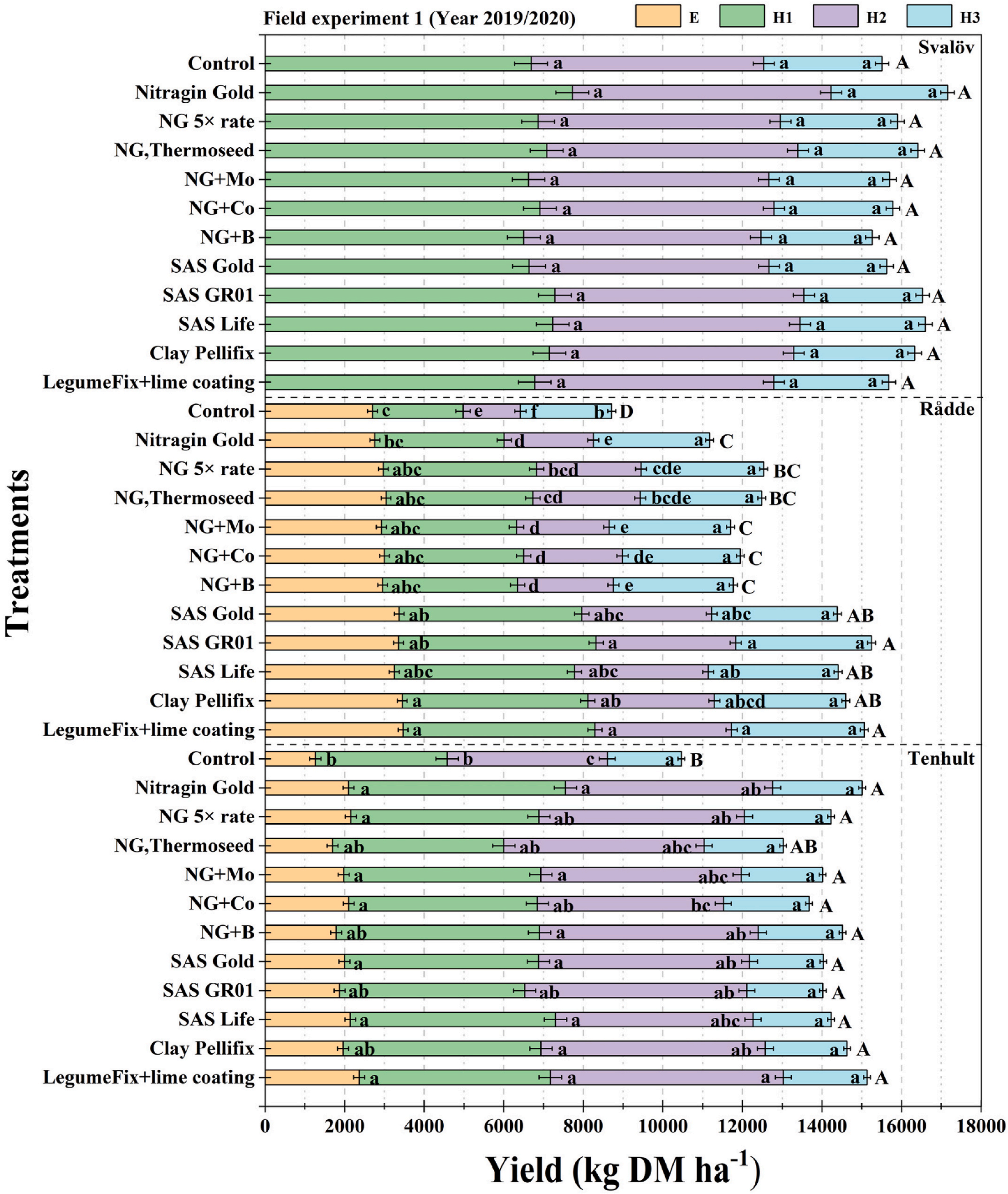

Fig. 1. Dry matter yield of lucerne in response to different treatments, at three sites in southern Sweden (year 2019/2020). E: establishment year. H: harvest. Means with different lower case letters indicate significant differences within sites and harvest at $P < 0.05$ according to Tukey's test. Means with different upper case letters indicate significant differences within sites for all harvests combined.

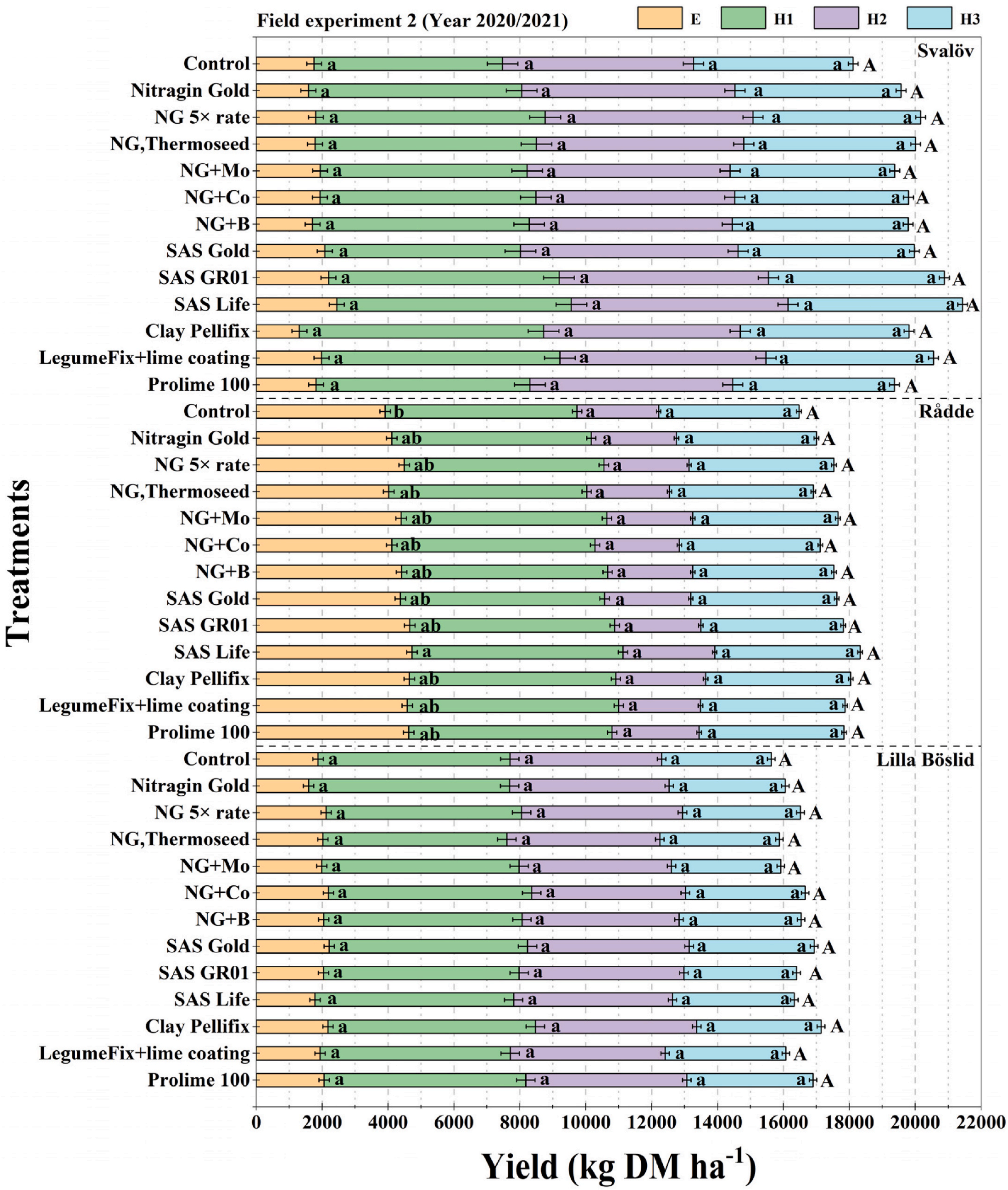

Fig. 2. Dry matter yield of lucerne in response to different treatments, at three sites in southern Sweden (year 2020/2021). E: establishment year. H: harvest. Means with different lower case letters indicate significant differences within sites and harvest at $P < 0.05$ according to Tukey's test. Means with different upper case letters indicate significant differences within sites for all harvests combined.

Table 5
Total nitrogen (TN) (% DM) of lucerne in response to different treatments in establishment and production years at three sites in southern Sweden (year 2019/2020). Means with different letters within columns indicate significant difference at $P < 0.05$ according to Tukey's test. E: establishment year. H: harvest.

Treatments		Svalöv	Rådde		Tenhult	
		H1	E	H1	E	H1
T1	Control	2.89a	1.72c	2.14c	2.88b	2.16b
T2	Nitragin Gold	2.87a	2.04abc	2.25bc	3.68a	2.47 ab
T3	NG 5 × rate	2.83a	1.98bc	2.37abc	3.81a	2.46 ab
T4	NG, Thermoseed	2.85a	1.81c	2.36abc	3.64 ab	2.47 ab
T5	NG + Mo	2.79a	2.12abc	2.19c	3.79a	2.57 ab
T6	NG + Co	3.01a	1.75c	2.25bc	3.67a	2.47 ab
T7	NG + B	2.87a	1.81c	2.27bc	3.62 ab	2.58 ab
T8	SAS Gold	3.00a	2.28 ab	2.54a	3.76a	2.62a
T9	SAS GR01	2.92a	2.33 ab	2.58a	3.69a	2.66a
T10	SAS Life	2.91a	2.46a	2.59a	3.70a	2.50 ab
T11	Clay Pellifix	2.98a	2.29 ab	2.48 ab	3.78a	2.47 ab
T12	LegumeFix + lime coating	2.94a	2.44a	2.57a	3.58 ab	2.70a
Mean		2.90	2.09	2.38	3.63	2.51
SEM		0.0676	0.0868	0.0530	0.152	0.0840
P-value		0.466	<0.001	<0.001	0.023	0.021

Table 7
Carbon nitrogen (C:N) ratio of lucerne in response to different treatments in establishment and production years at three sites in southern Sweden (year 2019/2020). Means with different letters within columns indicate significant difference at $P < 0.05$ according to Tukey's test. E: establishment year. H: harvest.

Treatments		Svalöv	Rådde		Tenhult	
		H1	E	H1	E	H1
T1	Control	15.6a	26.8a	15.9a	15.9a	21.2a
T2	Nitragin Gold	15.7a	22.8abc	12.2b	12.2b	18.3 ab
T3	NG 5 × rate	15.9a	23.4abc	11.8b	11.8b	18.3 ab
T4	NG, Thermoseed	15.8a	25.6 ab	12.4b	12.4b	18.4 ab
T5	NG + Mo	16.0a	22.0bc	12.0b	12.0b	17.5 ab
T6	NG + Co	15.0a	26.4 ab	12.3b	12.3b	18.3 ab
T7	NG + B	15.7a	25.6 ab	12.5b	12.5b	17.6 ab
T8	SAS Gold	15.0a	20.4c	11.9b	11.9b	17.3b
T9	SAS GR01	15.4a	20.2c	12.2b	12.2b	16.8b
T10	SAS Life	15.5a	19.1c	12.2b	12.2b	18.0 ab
T11	Clay Pellifix	15.1a	20.3c	11.9b	11.9b	18.2 ab
T12	LegumeFix + lime coating	15.2a	19.1c	12.6b	12.6b	16.6b
Mean		15.5	22.6	19.3	12.5	18.0
SEM		0.379	0.864	0.387	0.635	0.750
P-value		0.584	<0.001	<0.001	0.012	0.041

Table 6
Total nitrogen (TN) (% DM) and C:N of lucerne in response to different treatments in establishment and production years at three sites in southern Sweden (year 2020/2021). E: establishment year. H: harvest. "Other treatments" is an average of all other treatments besides the control.

Treatments	Svalöv	Rådde		Lilla Böslid	
	H1	E	H1	E	H1
TN					
Control	3.62	2.61	2.77	3.67	2.69
Other treatments	3.56	2.67	2.63	3.71	2.89
C:N					
Control	13.8	19.3	18.0	13.8	18.1
Other treatments	14.1	19.0	19.0	13.6	16.8

At Tenhult, all but two inoculation treatments had significantly greater NDVI than the control in the establishment year (2019) (Table 8). Four inoculation treatments (NG, NG + Co, SAS Life and LegumeFix + lime coating) had significantly greater NDVI than control at harvest 1 in the production year. None of the 11 inoculation treatments were significantly different from each other. There were no significant differences in NDVI among treatments or between treatments and the control at harvests 2 and 3. For the second experiment, there were no significant differences in NDVI between any treatments (Table 9).

4. Discussion

4.1. The effects of inoculations on lucerne establishment and production

Higher DM yield and TN concentration with the treatment compared to control during the first establishment/production cycle at Rådde and Tenhult with no lucerne history suggests that treatments improved the establishment of lucerne at these sites. There were no differences in DM yields and TN concentration between treatments and control at Rådde and Tenhult (no lucerne cultivation history) during the second cycle, and at Svalöv (with lucerne cultivation history) during both the first and second cycles. This suggests that there is a reduced influence of inoculants on plant growth where inoculants have previously been used, in line with the results of Stajkovic-Srbinovic et al. [13]. The possible reasons for the declining influence of inoculants in the second establishment/production cycle (2020/2021) are discussed in more detail in section 4.3.

The DM yields and TN concentrations were higher with five alternative inoculants than with standard NG and modified NG-related treatments and micronutrient treatments, particularly during experiment 1 at Rådde. Because all treatments used the same rhizobial strain, the differences in results between inoculants are likely due to the different formulations and methods of application to the seed [14,15]. The Pellifix product is a combination of rhizobia and clay, kept cold and moist until use, and then mixed with water to apply to seeds. The LegumeFix is a peat-based inoculant that was stuck to the seed with methyl cellulose glue, and then coated with fine lime. The three SAS products were similar to each other; they all included inoculum, nutrients and a finishing film. Although these five stand-out products included a range of inoculant types, their common factor leading to a positive plant response may be a high number of viable rhizobia on the seed; however this was not assessed in the current study. The benefits of these five alternative inoculant treatments may also be linked with soil characteristics [11,16]. High clay content in the soil has less influence of microbial inoculants on crop growth due to compaction and lack of aeration compared to soil with high sand content [17]. The efficiency of inoculants to establish nodules and initiate nitrogen fixation also depends on the interaction with the soil pH [18]. At Rådde, the soil clay content was the lowest and organic matter was the highest, which appeared to have created a better environment for these five alternative inoculants compared with soils at other sites.

In experiment 1, inoculation decreased C:N ratio at Rådde and Tenhult. The five alternative inoculant treatments, in general, had lower C:N ratios than other treatments. The low C:N ratio is opposite to the general pattern of the higher DM yield and TN with inoculant treatments compared to control at the sites. This suggests that the increase in N

Table 8
NDVI of lucerne in response to different treatments and harvests at Svalöv, Tenhult and Rådde in experiment 1 (year 2019/2020). E: establishment year. H: harvest. Means with different letters within columns indicate significant difference at $P < 0.05$ according to Tukey's test. E: establishment year. H: harvest.

Treatments		Svalöv				Rådde				Tenhult			
		E	H1	H2	H3	E	H1	H2	H3	E	H1	H2	H3
T1	Control	0.830a	0.843abc	0.843a	0.743a	0.747d	0.737d	0.760b	0.840b	0.730b	0.753b	0.857a	0.833a
T2	Nitragin Gold	0.830a	0.853a	0.847a	0.760a	0.773c	0.793bc	0.833a	0.857a	0.800a	0.823a	0.863a	0.843a
T3	NG 5 × rate	0.830a	0.843abc	0.840a	0.760a	0.797b	0.807abc	0.840a	0.857a	0.813a	0.817 ab	0.873a	0.840a
T4	NG, Thermoseed	0.823a	0.840bc	0.843a	0.767a	0.783bc	0.803abc	0.837a	0.857a	0.783 ab	0.803 ab	0.863a	0.827a
T5	NG + Mo	0.833a	0.847 ab	0.843a	0.760a	0.780bc	0.787c	0.830a	0.857a	0.793a	0.817 ab	0.860a	0.830a
T6	NG + Co	0.820a	0.847 ab	0.840a	0.760a	0.773c	0.790c	0.837a	0.853 ab	0.790a	0.827a	0.867a	0.837a
T7	NG + B	0.810a	0.833c	0.843a	0.760a	0.787bc	0.790c	0.823a	0.853 ab	0.777 ab	0.817 ab	0.863a	0.823a
T8	SAS Gold	0.827a	0.843abc	0.843a	0.760a	0.830a	0.827 ab	0.850a	0.860a	0.797a	0.817 ab	0.870a	0.820a
T9	SAS GR01	0.827a	0.847 ab	0.847a	0.760a	0.827a	0.830a	0.857a	0.860a	0.800a	0.813 ab	0.870a	0.827a
T10	SAS Life	0.823a	0.843abc	0.840a	0.757a	0.833a	0.830a	0.853a	0.860a	0.800a	0.823a	0.863a	0.833a
T11	Clay Pellifix	0.840a	0.847 ab	0.840a	0.763a	0.820a	0.820abc	0.850a	0.860a	0.803a	0.817 ab	0.863a	0.830a
T12	LegumeFix + lime coating	0.827a	0.843abc	0.840a	0.767a	0.837a	0.830a	0.857a	0.860a	0.817a	0.823a	0.860a	0.833a
Mean		0.827	0.844	0.842	0.760	0.799	0.804	0.836	0.856	0.792	0.813	0.864	0.831
SEM		0.00936	0.00207	0.00230	0.00696	0.00431	0.00666	0.0108	0.00274	0.0108	0.0126	0.00465	0.00581
P-value		0.791	<0.001	0.348	0.701	<0.001	<0.001	<0.001	0.002	0.002	0.037	0.434	0.266

Table 9
NDVI of lucerne in response to different treatments and harvests at Svalöv, Rådde and Lilla Böslid in experiment 2 (year 2020/2021). E: establishment year. H: harvest. "Other treatments" is an average of all other treatments besides the control.

Treatments	Svalöv				Rådde				Lilla Böslid			
	E	H1	H2	H3	E	H1	H2	H3	E	H1	H2	H3
Control	0.813	0.850	0.837	0.850	0.830	0.855	0.838	0.898	0.843	0.843	0.848	0.858
Other treatments	0.831	0.851	0.836	0.854	0.829	0857	0.833	0.895	0.849	0.842	0.844	0.866

relative C in DM yield was better with the treatments. Considering a low C:N ratio as an indicator of good forage quality [19], these results suggest that establishment inoculations can also improve forage quality. However, similar to the effect on DM yield and TN, the effect of treatments on C:N ratio is expected to diminish with time.

The effects of the inoculant treatment on NDVI were similar to their effects on dry matter yield and TN. As NDVI is affected by both plant biomass and greenness [20], these findings suggest that the positive outcomes of inoculants on dry matter yields and TN are also reflected in NDVI. This aligns with our previous study, where we demonstrated that the inoculants effects can be separated through NDVI [12]. Consistent with the trend observed in dry matter yield and TN, the effects of treatments, especially alternative inoculant treatments, were more pronounced in experiment 1 (year 2019/2020), at the sites without a history of lucerne cultivation (Rådde and Tenhult).

4.2. The effects of micronutrients on lucerne establishment and production

Lucerne has specific environmental requirements for growth and it is sensitive to low micronutrients (particularly B, Mo and Fe, but also Cu, Mn, Zn and Co) [10], so we hypothesised that "where soil test results indicate deficiencies in micronutrients, application of micronutrients can increase yield and nitrogen concentration of lucerne". However, this was not supported by the results. In this study, no added benefit of soil-applied micronutrients (B, Mo or Co) was observed, regardless of sites or years, even though some sites had low micronutrient values according to soil tests. Possible explanations for this phenomenon include (i) the requirement for these micronutrients is less than expected, (ii) the methods of applying micronutrients were ineffective, or (iii) the soil micronutrient analysis methods or their interpretations were not accurate. According to soil tests and interpretations provided by Eurofins, B was adequate at all sites, Mo was very low at all sites, and Co was low or very low at all sites except Rådde. Despite this, there was no evidence that any experimental plots were exhibiting B, Mo, or Co deficiency. In addition, the yields were generally high. It is unlikely that these yields (>15000 kg DM ha^{-1}) could be achieved if there was any micronutrient deficiency. The SAS Gold product contains micronutrients and inoculum, but it did not show any advantages over other SAS products in this study. The Legume Technology products, which contain no added micronutrients, performed as well as SAS Gold, suggesting that the method of adding micronutrients was not the issue. Therefore, micronutrients likely did not limit the establishment and production of lucerne in this study. Soil micronutrient concentrations and their interpretations were not a reliable indicator of nutrient deficiency, or whether lucerne would respond to soil-applied micronutrients.

4.3. The effects of sites on lucerne establishment and production

For areas in Sweden for which lucerne can be grown, it has the highest forage production potential of legume species, and where it is successfully established it persists well [21,22]. The ideal soils for lucerne growth should have adequate drainage, a suitable pH range and adequate macro and micronutrients. In our present study, the effects of cultural history on lucerne establishment and production were only observed in experiment 1 (2019/2020) – inoculation significantly improved lucerne yield and TN at Rådde and Tenhult, where lucerne had not previously been cultivated, whereas at Svalöv, where lucerne had been grown recently, there were no significant effects of inoculation. Although the experimental design in experiment 2 was similar to experiment 1, the reason for the lack of treatment effects is not clear. In experiment 2, the yield and TN for all treatments (even control) were higher compared to those in experiment 1. There are a number of potential reasons for this. Pre-existing lucerne Rhizobia in the experimental fields in experiment 2 may be an explanation for the lack of significant differences between treatments. It is important to emphasise that the experiments in cycle 2 were not established close to the experiments in cycle 1. The pH for Rådde and Lilla Böslid in experiment 2 was higher compared to the soil pH for Rådde and Tenhult in experiment 1. It is possible that the higher pH at these sites could have led to a longer-term survival of lucerne Rhizobia, which is known to be sensitive to pH.

Cross-contamination between plots is another phenomenon that may cause a non-significant difference between treatments, either through the sowing process or the flow of surface water across plots. In this series of experiments, we tried to control cross-contamination between plots by sowing control plots first. The surface flow of water is also unlikely to have been the cause because the spread of Rhizobia is more likely to occur when they have built up to high levels in the soil, a process which takes time and would be unlikely to happen in the establishment year. Experimental variability is also unlikely – in experiment 2, the number of replications was increased to 4, and there was no excessive variability between plots. At the sites where lucerne had previously been cultivated (Svalöv in this study), there was no effect of inoculation, neither in the establishment nor production year, for either experiment, suggesting that there is little value in inoculation where lucerne is known to have been successfully grown in recent years. However, the situation at Svalöv does not cover all possible scenarios, and there are unknown but conceivable interactions with soil organic matter content, micronutrients, and pH that could result in poor inoculation. Consequently, it seems risky to sow lucerne without inoculation, unless establishing in fields where lucerne has been grown successfully and recently. Quantifying the long-term effects of inoculants, while considering bacterial density dynamics, soil conditions, and climatic variability, could improve our understanding of their benefits and how long the bacteria persist in the soil.

5. Conclusions

This study demonstrated that alternative inoculants were better than NG and all NG-related inoculant or micronutrient treatments, particularly in the first production year at Rådde, where the best treatment yielded nearly twice that of control, and all alternative inoculant treatments had higher TN, lower C:N, and greater NDVI than the control. There was no added benefit of soil-applied micronutrients at any site in either year. Where there is a recent history of successful lucerne cultivation (Svalöv), lucerne could be well established and productive without inoculation of seed before sowing. However, the effects of inoculation vary between different fields and years and therefore, seed inoculation could serve as an insurance for a well-established and productive lucerne crop.

CRediT authorship contribution statement

Lin Tang: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Uttam Kumar:** Writing – review & editing, Formal analysis. **Linda Öhlund:** Writing – review & editing, Investigation. **David Parsons:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2025.101644.

Data availability

Data will be made available on request.

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