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Project Report

Evaluating Effects of *Bradyrhizobium* and Arbuscular Mycorrhizal Fungi Inoculation on Yield Components of Mung Bean (*Vigna radiata* (L.) Wilczek) and Nitrogen Fixation

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Abstract: Mung beans (*Vigna radiata* (L.) Wilczek) are only inoculated in some production systems, but there is a current lack of knowledge on the best inoculants to use for effective nitrogen fixation (nodulation) and plant yields. The objectives of the present study were to determine if the dual inoculation of Arbuscular Mycorrhizal Fungi (F) and *Bradyrhizobium* (R) provides greater (a) mung bean yield and quality (b) nitrogen fixation for mung bean and residual soil nitrogen for the following crop, and (c) determine if these effects are consistent across various environments. Field trials were conducted in Blacksburg, VA (sandy clay loam), and Eastern Shore, VA (sandy loam), over the summers of 2020 and 2021. There were 5 treatments replicated 5 times for each variety at each site; R, F, R + F, high nitrogen (N) (100 kg ha^{-1}), and a control, for a total of 25 plots per site. Mung beans grown in Blacksburg in 2020 and 2021 averaged 53.8% more seeds per pod than mung beans grown at the Eastern Shore. Overall yield components (seeds per pod, pods per plant) are heavily influenced by soil type. Dual inoculation significantly increased grain yield (+33%) compared to a synthetic N fertilizer application, but did not significantly increase grain yield compared to the control (+22%). Dual inoculation may increase the grain yields of mung beans compared to synthetic fertilizer regime, but does not show evidence of improving N fixation.

Keywords: mung beans; inoculation; Arbuscular Mycorrhizal Fungi; soil fertility

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1. Introduction

Mung bean (*Vigna radiata* (L.) Wilczek]) also known as green gram or moong, is a warm-season pulse legume with a short growing season (50–70 days) that is relatively drought-tolerant and well acclimated to sandy soils with poor fertility [1,2]. Mung bean is widely considered to be native to tropical and sub-tropical regions of India [2] but is grown in most tropical and sub-tropical regions as well as in temperate climates globally. In addition to being a rich source of protein, mung bean is also high in folate, which is an essential nutrient for pregnant and nursing women as well as an excellent source of calcium and phosphorus, containing 118 mg Ca per 100 g of seed and 340 mg P per 100 g of seed [2]. Currently, mung bean production in the United States is largely focused in the state of Oklahoma, growing just over 45,000 hectares for forage and grain. The use of mung bean as a specialty crop has grown considerably in the past 5 years due to increases in demand for plant-based meat alternatives in the United States [3]. Mung bean can be grown under rain-fed conditions, in well-drained soils with optimal temperatures between 20 and 40 °C, making it a viable, low-input solution to late-season forage gaps experienced in the mid-Atlantic geographic region and a potential cash crop in anticipated markets [4].

In order to optimize legume production, producers are encouraged to use plant growth-promoting rhizobacteria (PGPR) or proper 'inoculant'. With proper bio-fertilization (inoculation), nitrogen fixation is enhanced in leguminous crops. N_2-fixing pulse crops can acquire an average amount of 140 kg N ha^{-1}, with about 18–30% of that being left for the next crop depending on the soil management, texture, and climate [5,6]. The use of biofertilizers in pulse crop production can be a cost-effective and environmentally friendly alternative to synthetic fertilizer production [7]. Some factors affecting proper nodulation include, soil acidity, the water holding capacity of the soil, and soil texture [8,9]. Soil transfer, or residual indigenous rhizobium, is largely considered to be the first method of legume inoculation and early experiments of exogenous soil inoculation saw yields 10 times that of the control [10]. Applications of both *bradyrhizobium* (R) and Arbuscular Mycorrhizal Fungi (F) have been shown to significantly increase grain yield in mung bean production compared to a control [11]. Field crop legumes that make up a majority of global production are inoculated by the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *or Bradyrhizobium* and are referred to as symbionts. Arbuscular Mycorrhizal Fungi (F) also form symbiotic relationships with legumes via similar mechanisms as rhizobium, collecting mainly P and other nutrients for the plants to use [12,13]. Arbuscular Mycorrhizal Fungi are not limited to interactions with legumes, as they have been shown to colonize 80% of plant species [14]. The organisms will typically colonize and extend root systems of plants which could allow for increased overall nutrient uptake [15,16]. "The symbiosis enhances the ability of the plant to become established and cope with stress situations (nutrient deficiency, drought, trace element imbalance, soil disturbance), which are typical in desert situations" [11]. Plants that have been colonized by Arbuscular Mycorrhizal Fungi have also shown to improve total plant chlorophyll levels, photosynthesis rates, and transpiration rates [17].

The cultivar of mung selected for growth has a significant effect on yield and nodulation, with hybrids typically out performing local varieties although this effect has not been studied much further [18,19]. Many studies have shown that *rhizobium* inoculation can significantly increase yield components of mung bean such as number of pods, seed weight, and plant residue [8,20,21]. Seed inoculation along with P application of 50 kg ha^{-1} has been shown to be the most efficient use of resources and produce greatest grain yields although specific inoculant is not explicitly addressed [22]. Anjum et al. (2006) found that seed inoculation was more effective compared to soil inoculation when observing yield parameters in mung bean [20].

Currently, there is a lack of knowledge pertaining to growing mung beans in Virginia and its possible economic and/or environmental impacts. Few field trials have investigated the effect of the dual inoculation of *bradyrhizobium* and Arbuscular Mycorrhizal Fungi on mung bean yield and yield components, while also observing residual soil fertility across two environments. Legumes such as alfalfa, and various clovers have proven to be effective, high-quality forages for animal production in Virginia [23,24]. At Virginia Tech, mung bean has been explored as an alternative forage legume for its low input and stress tolerance. We hypothesize that the combination of Arbuscular Mycorrhizal Fungi and *rhizobium* inoculum in mung bean production will improve the yield components of mung bean while also increasing the rate of N-fixation, increasing overall efficiency of production systems and providing key insights into the future of mung bean management and production in Virginia.

2. Materials and Methods

2.1. Experimental Design

Field plots were established at the Eastern Shore AREC, in Painter, VA (37.587399, −75.824835) and the Homefield Farm in Blacksburg, VA (37.203515, −80.565049) during the summers of 2020 and 2021 in portions of land that had no previous inoculum over the past 7 years. Each field site had an equal number of plots and replications (5) at each site. The variety of mung bean used was "Berken", purchased from Oklahoma Foundation Seed Stocks in Stillwater, OK. "Berken" is widely grown in the United States. Plots were established in a randomized complete block design (RCBD). The experiment utilized 5 treatments: R, F, R + F, high N 100 kg N ha^{-1} (N), and a control (C). Thirty seeds were planted per row with 4 rows per plot application at 3.05 m × 3.05 m long. There was no irrigation utilized for the duration of the present study.

Each site was carefully managed for weeds with routine applications of post emergence herbicides Clethodim 2e® and Basagran® at the manufacture recommended rates of 1.2 L ha^{-1} and 2.3 L ha^{-1}, respectively.

2.2. Soil Description

The soil in Blacksburg, VA was well-drained Ross loam soil, with 2% slopes. The Eastern Shore AREC site was Bojac Sandy loam soil. The Painter site was previously in tomato production and non-leguminous cover crops while the site in Blacksburg was under cover crop and melon rotation. We did not suspect the presence of foreign symbiotic bacteria due to no legumes in previous rotations and the soil was not tested for any naturally occurring bacteria that would affect results of this study.

2.3. Plant Nutritional Analysis

Five plants from each plot were randomly selected and hand harvested once to record grain yields (number of seeds, seed weight, and pods per plant). The same plots used for yield components were analyzed for crude protein content as well as dry-matter above-ground biomass yields, and plant height [25]. For dry matter determinations, plants selected for harvest were oven dried at 50 °C to a constant weight and ground for further analysis.

2.4. Rhizobium and Mycorrhizal Fungi Inoculation

Before sowing, mung bean seeds were inoculated with *Bradyrhizobium* inoculum, at a rate of 10 g inoculant kg^{-1} according to package instructions, purchased from Hancock Farm & Seed Co., Inc. (Dade City, FL, USA). The *rhizobium* inoculant contained *Rhizobium leguminosarum biovar viceae*: 2×10^8 CFU/g, *Bradyrhizobium* sp. (Vigna): 2×10^8 CFU/g, and *Rhizobium leguminosarum biovar phaseoli*: 2×10^8 CFU/g. "Myco Bliss" granular powder (Arbuscular Mycorrhizal Fungi inoculant) was purchased from Plantonix, LLC. (Ashland, OR, USA) and applied at a rate of 2.2 kg ha^{-1} per acre, in row, at planting according to manufacturer instructions. The formulation contained equal portions of 5 species of *endomycorrhizal inoculants*, *Rhizophagus irregularis*, *Rhizophagus aggregatus*, *Rhizophagus proliferum*, *Rhizophagus clarus*, and *Claroideoglomusetunicatum*, at 175 propagules g^{-1}. Nodulation by *bradyrhizobium* were analyzed by direct observation. Selected plants were excavated and carefully washed at 30 days after sowing and at maturity, approximately 100 days after sowing (DAS). Nodules were observed using a magnifying glass [26]. Results are not reported here due to lose of nodules during plant excavation.

2.5. Soil Fertility Analysis

Routine soil analysis was conducted pre-planting and post-harvest to measure N concentrations in the soil. The determination of soil ammonium (NH_4^+) and nitrate (NO_3^-) was made using Lachat QuikChem AE flow-injection autoanalyzer and ion chromatography. The soil samples were air dried, ground and passed through a 10-mesh (2-mm) sieve [27]. Mehlich 1 extracted P was determined at a ratio of 5 g soil: 20 mL of extraction solution containing 0.05 N HCl and 0.025 N H_2SO_4. [28]. Soil pH was determined using a 1:1 (*v*/*v*) soil-water mixture [29]. Soil physical and chemical property data from locations and both years are displayed in Table 1. Soil pH for was considered to be optimal for successful mung bean growth. High soil phosphorus levels were observed at the Eastern Shore for both 2020 and 2021. Levels of soil K, Ca, and Mg were considered to be optimal for efficient mung bean growth.

Table 1. Summary of soil chemical properties at Blacksburg and Eastern Shore sites in 2020 and 2021.

Year	Location	pH	NO_3^-	NH_4^+	P	K	Ca	Mg	SOM
			mg kg^{-1}			mg kg^{-1}			%
2020	Blacksburg	7.1	14.9	8.7	10.0	88.0	680	180	5.2
	Eastern Shore	6.5	16.6	5.0	46.2	136	392	44	1.0
2021	Blacksburg	6.9	17.4	5.2	15.2	150	744	189	4.9
	Eastern Shore	6.4	5.6	7.1	44.0	130	372	54.0	1.5

2.6. Statistical Analysis

Statistical analysis was performed using yjr RStudio software (version 3.2.2) (RStudio Team, 2015). Two-way ANOVA was conducted by plot location and cultivar for each of the treatments to investigate soil N availability in the soil with significance indicated by p-values < 0.10. All treatment differences were investigated using post hoc Fisher's Least Significant Difference (LSD) at a significance level of $p < 0.10$. All yield data collected from the Eastern Shore site during 2020 were excluded due to excessive crop damage and delayed maturity.

3. Results and Discussion

3.1. Plant Height

The comparison of location on plant height showed that plants grown on the Eastern Shore were, on average, taller than plants grown in Blacksburg in both 2020 and 2021 (Table 2). Mung bean plants grown at the Eastern Shore in 2021 (96.9 cm) were, on average, 16.9% taller than plants grown in Blacksburg during 2020 (83.54 cm) and 24.6% taller than plants grown in Blacksburg during 2021 (77.2 cm) (Table 2). These results are consistent with Diatta et al. (2018), who reported differences in mung bean plant height when grown in different soil textures (sandy loam and a loam soil) [10]. The significant differences in height, as related to soil texture, may also be attributed to seasonal temperature differences as well as precipitation during the season. Average monthly temperatures in Blacksburg were consistently lower than average monthly temperatures on the Eastern Shore (4–5 °C lower). Consistent differences in mung bean physiology have been observed, noting significant decreases in stem length as temperature decreased [30]. A study evaluating precipitation effects on other legumes, found that a reduction in precipitation led to accelerated maturity and decreased plant height [31]. While total precipitation at both locations during both seasons was relatively similar (Table 3), the timing of that precipitation may have influenced the overall height of the plants each year. The plants grown at the Eastern Shore during 2021 received 56% more precipitation during July compared to plants grown during 2020 in Blacksburg (150 mm vs. 66 mm) and 52% more precipitation than plants grown during June 2021 (121 mm vs. 58 mm). Significant differences in precipitation at planting may have influenced overall plant height differences reflected at each location despite soil texture differences.

The location by treatment interaction was statistically significant for plant height (Table 4). Plants grown in Blacksburg during 2020 and at the Eastern Shore in 2021 showed no significant difference in plant height when considering treatment, alone (Table 2). The height of plants treated with the N fertilizer were, on average, taller than plants grown with the *Bradyrhizobium* spp. inoculant (85.7 cm vs. 70.7 cm). These results are contrary to a similar study reporting that *Bradyrhizobium* spp. inoculant increased plant height when compared to an N fertilizer treatment [32]. This may be explained by the different rate and source of N fertilizer used in their study and optimal soil nutrient levels when considering soil P, K, Ca, and Mg (Table 1) in the Blacksburg 2021 soil.

Table 2. Mungbean plant height, dry biomass, seeds per pod, and pods per plant by treatments at Blacksburg 2020, Blacksburg 2021, and Eastern Shore 2021. (n = 225 plants).

Location	Control (c)	Arbuscular Mycorrhizal Fungi (F)	Nitrogen Fertilizer (N)	*Bradyrhizobium* (R)	R + F
Plant height (cm)					
Blacksburg 2020	84.7 a	83.1 a	82.7 a	86.4 a	80.7 a
Blacksburg 2021	73.5 ab	77.4 ab	85.7 a	70.7 b	78.5 ab
Eastern Shore 2021	97.5 a	101.6 a	89.1 a	91.4 a	104.8 a
Dry biomass (g)					
Blacksburg 2020	131.8 a	141.4 a	121.2 a	118.6 a	124.2 a
Blacksburg 2021	115.5 b	160.9 a	132.9 ab	152.4 ab	159.8 a
Eastern Shore 2021	128.5 ab	133.8 ab	118.2 b	171.2 a	134.2 ab
Seeds pod^{-1}					
Blacksburg 2020	10.8 a	10.7 a	10.9 a	11.2 a	11.9 a
Blacksburg 2021	9.7 a	10.5 a	10.2 a	10.2 a	9.5 a
Eastern Shore 2021	7.0 a	8.1 a	6.5 a	6.9 a	6.6 a
Pods plant^{-1}					
Blacksburg 2020	61.8 ab	47.2 b	49.3 ab	62.7 ab	70.8 a
Blacksburg 2021	20.2 a	29.4 a	21.9 a	37.6 a	30.4 a
Eastern Shore 2021	12.0 a	13.5 a	8.2 a	16.2 a	12.7 a

Means within each row followed by different letters are significantly different according to Fisher's protected LSD (α = 0.10).

Table 3. Monthly rainfall (mm) and temperature (°C), between June and November at Blacksburg and Eastern Shore sites in 2020 and 2021.

Year	Location	Parameter	June	July	Aug	Sept	Oct	Nov	Total
2020									
	Blacksburg	Rainfall (mm)	127	66	183	117	100	137	729
		Temperature (°C)	20	24	22	17	13	8	
	Eastern Shore	Rainfall (mm)	84	81	188	163	112	141	768
		Temperature (°C)	23	28	26	22	17	13	
2021									
	Blacksburg	Rainfall (mm)	58	178	118	184	81	24	643
		Temperature (°C)	20	22	23	18	15	5	
	Eastern Shore	Rainfall (mm)	121	150	181	84	78	-	613
		Temperature (°C)	24	26	26	22	19	-	

Table 4. Analysis of variance of site, treatments, their interactions, and block for yield and yield components of mung bean and difference in soil N as nitrate and ammonia, from planting to harvest.

Source		Plant Height	Dry Biomass	Seeds	Number Pods	Soil Nitrate	Soil Ammonia	Grain Yield
	df	cm	g Plant^{-1}	Pod^{-1}	Plant^{-1}	mg kg^{-1} Plot^{-1}		Kg ha^{-1}
Location	1	0.0001	0.1477	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment	4	0.4583	0.1049	0.9441	0.1045	0.0698	0.5223	0.1484
Location × Treatment	4	0.0734	0.2087	0.9345	0.6030	0.2063	0.2904	0.4107
Block	4	0.3638	0.0624	0.0008	0.5410	0.2578	0.3322	0.3607
Model	13							
Error	61							
C. Total	74							

3.2. Dry Biomass

While the overall ANOVA for above ground dry biomass was not significant, treatment effects were observed in 2021 at both the Eastern Shore and Blacksburg locations (Table 4). In Blacksburg, the F treatment produced, on average, 39.1% more biomass than the control (115.5 g plant^{-1} vs. 160.9 g plant^{-1}) and the *Bradyrhizobium* + F dual inoculation treatment produced 38.2% more biomass than the control (159.8 g plant^{-1} vs. 115.5 g plant^{-1}). This is consistent with findings from Uddin reporting that overall plant biomass of mung beans was significantly increased by the use of bio-fertilizers when compared to a control treatment [32]. These results are most likely a function of the availability of nutrients provided by the symbiotic relationships. At the Eastern Shore (2021) location, the *Bradyrhizobium* treatment produced 44.8% more biomass, on average than the nitrogen fertilizer treatment (171.2 g plant^{-1} vs. 118.2 g plant^{-1}). While there are significant differences between treatments in some cases, it should be noted that these differences were not consistent across years or location.

3.3. Pod Number and Seeds per Pod

The number of seeds per pod was significantly affected by location (Table 4). Mung beans grown in Blacksburg (sandy clay-loam) in 2020 and 2021 averaged 53.8% more seeds per pod than mung beans grown at the Eastern Shore (sandy loam) in 2021, regardless of treatment (Table 2). This finding is consistent with results that were reported by Oke and Eyitayo, considering soil texture and fallow effects on cowpea (*Vigna unguiculata*) pod development [33]. They reported an increase in overall pod development in a sandy clay loam over a sandy loam. The difference across soil type may be due to several factors including planting date, higher pH, and greater percentage of exchangeable cations, as seen on Table 2. Higher percentages of water retention on the sandy clay loam may also be a factor in the increase in seeds per pod. When considering temperature differences (Table 3), reported consistent results noting that a 2–3 degree increase in temperature can significantly decrease overall yield (−13.6%) and inhibit pod formation due physiological stress [34].

The number of pods per plant was significantly affected by the location of the mung beans (Table 4). Mung beans planted in Blacksburg in both 2020 and 2021 produced 68.3% to 490% more pods per plant compared to those planted at the Eastern Shore in 2021 (Table 2). The overall increase in pods per plant by location can be attributed to several factors such as planting date, higher pH, and greater percentage of exchangeable cations, as seen on Table 1. These results are consistent with previous findings from Oke and Eyitayo with soil texture and location greatly influencing yield components such as pods per plant [33]. Increased nutrient concentrations in the soil and optimal timings of precipitation events may have contributed to the location effect on the number of pods per plant and seeds per pod.

3.4. Soil Nitrogen

Differences in residual soil nitrate at harvest were significantly affected by the location and treatment. Among the treatments at the Blacksburg locations in 2020 and 2021 only the R + F treatment during 2021 produced a significantly smaller amount of nitrate as compared to the rest of the treatments (Figure 1a). Similar results were found in Australian mung bean production systems, where inoculation resulted in little change to residual soil nitrate differences at harvest as well as %Ndfa (Nitrogen Derived From the Atmosphere) [35]. The study suggests that current inoculation practices are not effective enough resulting in poor nodule activity. In the present study, on average, plots treated with R or R + F, had less residual nitrate in the soil across locations, even though this difference was not statistically significant. Soil nitrate levels at the Blacksburg location for both years were significantly higher than plots at the Eastern Shore during 2021. The organic matter concentration of the soil in Blacksburg was significantly higher than in the eastern shore (Table 1) which explains some of the stark differences in soil nitrate. Reductions in nitrate leaching has been shown in soils with higher organic matter concentrations [36].

Differences in residual soil ammonia at harvest were only significant when comparing across locations (Figure 1b). Overall levels of soil ammonia were significantly lower in Blacksburg during 2021. The effect seen here may be explained by rapid ammonium volatilization and nitrification due to the environmental conditions and soil health of Blacksburg versus the conditions at the Eastern Shore. Differences in soil texture, nutrient holding capacity, and volatilization rates may have led to this difference in ammonia concentration in the soil. The treatments did not have a clear effect on the ability of mung beans N fixation capabilities.

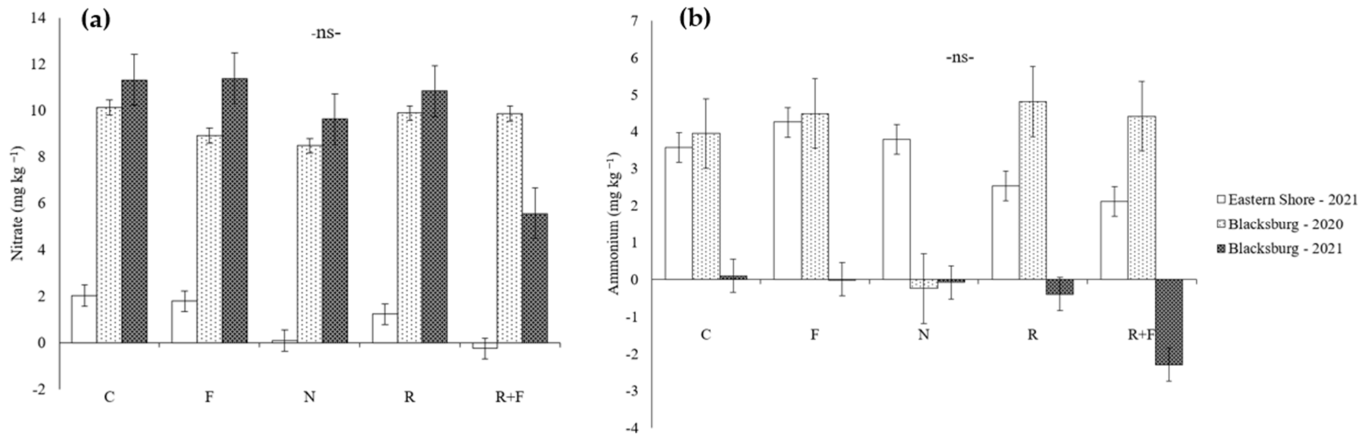

Figure 1. (**a**) Average difference in soil nitrate, from planting date until harvest, across treatments at each location. (**b**) Average difference in soil ammonia, from planting date until harvest across treatments at each location, at harvest. Treatments are labeled as follows: C = control, F = *Arbuscular Mycorrhizal Fungi*, N = Urea fertilizer, R = *Bradyrhizobium* spp., R + F = Dual inoculation with *Bradyrhizobium* spp. and Arbuscular Mycorrhizal Fungi. Treatments connected by different letters are significantly different at $\alpha = 0.10$ according to Fishers protected LSD. (n = 75). (**a**,**b**) are denoted by "ns", meaning treatment differences within each location are not significantly different.

Treatment comparisons within each location were not statistically significant. However, at the Eastern Shore location, we did note a decline in soil nitrate and ammonium levels when comparing the F treatment to the R or R + F treatment. This may suggest the viability of Arbuscular Mycorrhizal Fungi inoculant, but further confirmatory analysis would need to be conducted.

3.5. Grain Yield

Figure 2a shows the combined yields from each year and location. The dual-inoculation treatment produced a similar amount of grain compared to the control, F, and R solo treatments. The dual inoculation and *Bradyrhizobium* solo treatment yielded approximately 45% more grain than the urea fertilizer treatment. These results are consistent with previous studies finding that excess N fertilizer does not correlate to increased grain yields and can inhibit the nodulation within legume crops [9,37,38].

The comparison of location on the average grain yield of mung bean was significant. Our data showed that plants grown in Blacksburg in both 2020 and 2021 produced more grain than plants grown at the Eastern Shore in 2021 (Figure 2b). The R + F treatment in Blacksburg during the 2020 season produced the max grain yield (3728 kg ha^{-1}) which was 25.9% more than the control and that same year 67.4% more than the F treatment alone in that same year. These findings are similar to the grain yield trends for mung bean reported by Havugimana et al. [11] when comparing dual inoculation of rhizobia and F to a control and solo treatments. They reported a 24–44% increases in grain yield with the dual-inoculation treatment compared to the control and F solo treatments [11].

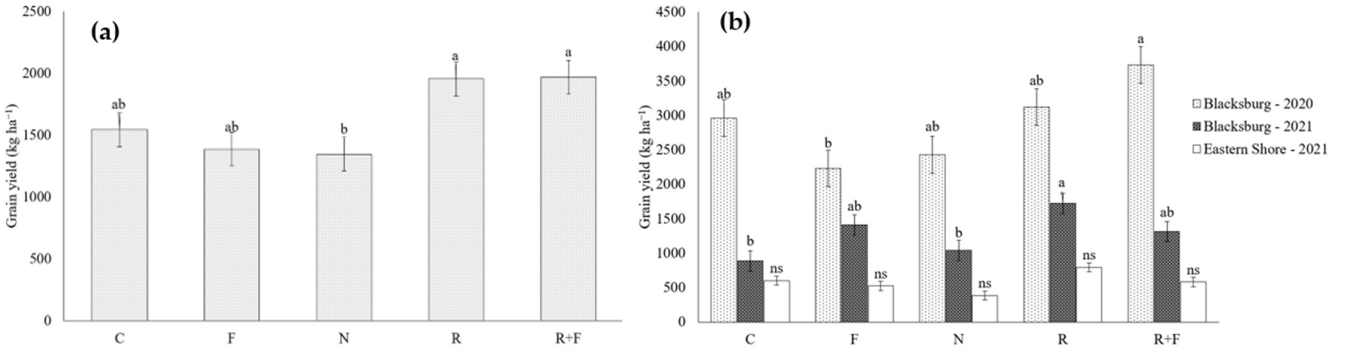

Figure 2. (**a**) Average grain yields from both locations and years by treatment. (**b**) Grain yields from each location by treatment. Mean comparisons are made between treatments at the respective location. Error bars represent the standard error for observations at each location. Treatments are labeled as follows: C = control, F = Arbuscular Mycorrhizal Fungi, N = Urea fertilizer, R = *Bradyrhizobium* spp., R + F = Dual inoculation with *Bradyrhizobium* spp. and Arbuscular Mycorrhizal Fungi. Treatments connected by different letters are significantly different at $\alpha = 0.10$ according to Fishers protected LSD (n = 75). Treatment differences that are not significantly different are denoted by "ns" above the error bar.

4. Conclusions

Overall, mung bean yield components (plant height, number of seeds per pod, pods per plant, and grain yield) were heavily influenced by location. The dual inoculation of mung bean with *Bradyrhizobium* + F or just *Bradyrhizobium* seems to be a promising agronomic practice for increasing grain yield compared to using a synthetic nitrogen fertilizer but is not statistically an improvement compared to the control. Nitrogen fertilizer amendments seem to inhibit pod formation and reduce residual soil ammonium at harvest while increasing total biomass. *Bradyrhizobium* inoculation decreased plant height, which could reduce lodging and disease formation on plants. Inoculation also increased plant biomass, which could prove to be a benefit in forage and cover cropping systems where ground cover is an important factor for successful production. The significance of location to almost every factor may be a result of the elevated soil organic matter concentrations in Blacksburg and a generally more suitable growing season, making Western Virginia a viable location for mung bean production. These results, indicating significant effects of location and treatment on mung bean yield and yield components, need to be further analyzed through more field experiments. F colonization studies and nodulation studies in our geographic region could add more clarity to these preliminary results.

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