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The influence of biofertilizer effect on switchgrass (*Panicum virgatum*) crop yield under greenhouse and field conditions in Guelph, Ontario, Canada

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ABSTRACT

Greenhouse and field experiments were conducted to test the effectiveness of biofertilizers on switchgrass (*Panicum virgatum*) yields compared to inorganic fertilizer and a zero-control. In the greenhouse, *Variovorax paradoxus* JM63, JumpStart® (*Penicillium bilaii*), inorganic fertilizer and control treatments resulted in significantly higher per L pot biomass yields compared to the control treatment; 2.74 (±0.24), 2.55 (±0.10), 2.52 (±0.24) and 1.34 (±0.09) g L-1, respectively. As JumpStart® is a commercially available biofertilizer, it was used in the field experiment along with inorganic and control treatments. All three treatments were applied to established (2014) switchgrass plots. Significantly ($p<0.05$) higher biomass yields of 10.73 (±1.33) and 7.67 (±0.30) Mg ha-1 were recorded for JumpStart® and inorganic fertilizer treatments, respectively, when compared to the zero-control biomass yield of 5.36 (±0.87) Mg ha-1. The enumeration soil test revealed that soil from JumpStart® and zero-control treatments had 20033, and <100 cfu/g of soil of *Penicillium spp.*, respectively. Results suggest that commercially available JumpStart® could replace/supplement inorganic fertilizer application on well-established switchgrass fields but, its influence on long-term biomass yields need to be further verified.

Keywords: Herbaceous biomass, soil health, Rhizobacteria, Penicillium bilaii

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Introduction

North America is the leading region in the world for inorganic fertilizer use that contributes to greenhouse gas (GHG) emissions from agricultural production[1]. Environmental problems associated with excess inorganic fertilizer use include phosphorous loading to waterways leading to algae blooms and increased nitrous oxide emissions[2,3]. Fertilizers can be an effective tool to enhance crop productivity but are often exploited to obtain desired results[2]. North America represents 13.5% of global fertilizer use and has been projected to increase 0.4% annually[1]. The agricultural sector in Ontario, Canada steadily contributes GHG emissions since 1990, averaging 10 Mg CO_2e per year[4,5]. Therefore, finding a suitable replacement for inorganic fertilizer use in the current production systems is fundamental towards reducing negative environmental consequences not only in Ontario but globally.

Plant growth promoting rhizobacteria (PGPR), or biofertilizers, can potentially be a viable solution to increase crop yields with less negative impacts on the environment, particularly GHG emissions. Biofertilizers are applied to seeds, plant surfaces or soil and have the ability to colonize in the rhizosphere or interior of the plant and will promote growth by increasing the supply of available primary nutrients for the host plant[6]. *Variovorax paradoxus* JM63 and JumpStart® (*Penicillium billai*), solubilize phosphate minerals making them readily available in a form that the host plant can uptake then use for enhanced plant growth[6,7].

The common mode of action of *V. paradoxus* JM63 and JumpStart®, is solubilizing unavailable nutrients, especially phosphorus. It has been stated in the literature that only 0.1% of available phosphorous is accessed by plants[8]; plants can only take up phosphorous that is in monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) solubilized forms[9]. *V. paradoxus* JM63 and JumpStart® are able to solubilize inorganic phosphates by the release of organic acids, 2-ketogluconic acid and gluconic acid, or mineralize organic phosphates by depositing extracellular phosphatases[10]. Vessey (2003) states that in order for PGPR to be effective in relation to plant growth and development, an intimate association between the PGPR (biofertilizer) and the host plant is required.

In Ontario, Canada biomass crops have become an interest to be used as a source of renewable advanced biofuels, combustion for heating or electricity, the oils used for bioplastics, and animal feed or bedding[11,12]. The most common biomass crop grown in Ontario, Canada is switchgrass (*Panicum virgatum*) and producers maintain fertilization of their biomass crops to sustain higher crop yields. Switchgrass is a perennial crop that has a long lifetime that can maintain yields for 10-20 years[13]. Other biomass crops grown in Ontario, Canada are Miscanthus (*Miscanthus* spp.), hybrid poplar (*Populus* spp.), and hybrid willow (*Salix* spp.)[13]. Therefore, this study seeks to accomplish the following objectives: (1) To test biofertilizer treatments in a greenhouse study on switchgrass and quantify yield response between treatments, (2) To evaluate biofertilizers on a 4-year-old crop of switchgrass (cultivar: 'Cave-in-Rock') under field conditions at the Guelph Turfgrass Institute (GTI), Guelph, Ontario, Canada.

Methodology

Greenhouse experiment

Greenhouse (University of Guelph, Guelph, ON, Canada) experiment was setup in a randomized complete block design (RCBD) using Elite 300 pots (New Christie Ventures, Naugatuck, Connecticut). Elite 300 pots (6.5 inch in diameter, 3 litres capacity) were used to ensure that growth can be maximized by not limiting root growth. The blocks were setup perpendicular from the potential environmental gradient, in this case the door to the greenhouse. Switchgrass cultivar 'Cave-in-Rock' seed was supplied by Eggimann Farms (Holland Centre, ON, Canada). Due to low germination test results (19% germination), seeds were pre-germinated in the laboratory and then transplanted into the Elite 300 pots. Seeding rate (seedlings) was 45

kg ha^{-1}, which translated into 54 plants per pot. Results for biomass yields are reported in g L^{-1} of the pot volume. Soil used in the pots for greenhouse experiment was taken from the GTI field in order to maintain the same soil medium; sandy loam (52% sand, 43% silt, 5% clay).

V. paradoxus JM63 was applied at a concentration of $1x10^8$ CFU ml^{-1} and JumpStart® was applied at a concentration of $7.2x10^8$ CFU ml^{-1} of mixed solution (distilled water) at the rate of 2 ml per seed corresponding to rates of 3.5 L per 1135 Kg of seed, as per product description and literature[6,14]. Inorganic fertilizer (16-16-16) was applied at 60 kg N ha^{-1} [15]. Each pot (Elite 300 pot) received 7 g of fertilizer. All treatments were applied after gemination.

Inoculum methods were adapted from Vessey (2003) and Dashti *et al.* (1997). *V. paradoxus* JM63 was cultured in lysogeny broth medium until it contained $1x10^8$ CFU ml^{-1}. This was determined by measuring the optical density of the inoculum at set times and associating the reading with growth curve results. *V. paradoxus* JM63 was grown in lysogeny broth at 30°C and shaken at 115 revolutions per minute[6,16]. The treatment was applied on the same day once the culturing and predetermined optical density reading was achieved.

The environmental conditions that were maintained in the greenhouse were: 1) temperature at 25°C, and 2) lights on for 16 hours per day[16]. This allowed for optimal growing conditions for switchgrass and plants were watered as needed[17], and the amount of water applied to each pot was kept equal.

Treatments

The treatments in the Greenhouse experiment were: 1) *Variovorax paradoxus* JM63, 2) *Penicillium bilaii* (JumpStart®), 3) Inorganic Fertilizer and 4) zero-control.

As biofertilizer and inorganic fertilizer treatments gave similar but significantly higher biomass yields compared to the control treatment in the greenhouse study, the biofertilizer JumpStart® was selected for the field study as this biofertilizer is commercially available and can be accessed by the biomass growers. Therefore, the treatments in the Field experiment were: 1) *Penicillium bilaii* (JumpStart®), 2) Inorganic Fertilizer, and 3) zero-control. These three treatments were applied in the spring of 2018 and then harvested in the autumn (2018).

Field experiment

The field experiment was implemented on switchgrass plots that were previously established in the summer of 2014 at the GTI, Guelph, Ontario, Canada. Each treatment area was 1 m^2, with 3 m buffer between each treatment within a block (10m x 20m), replicated 4 times. Switchgrass cultivar examined was 'Cave-in-Rock', the treatments investigated were JumpStart®, inorganic fertilizer, and zero-control. JumpStart® was applied at a concentration of $7.2x10^8$ cfu/ml of mixed solution (distilled water) at the rate of 16 ml/m^2. The application rate of JumpStart® was 3.5 L per 1135 Kg of seed, as per product description[14] and inorganic fertilizer (16-16-16) was applied at 60 kg N ha^{-1} [15]. JumpStart® application was based on a seeding rate of 30 kg ha^{-1}, which was used in 2014 during the time of establishment.

Plant Sampling

Plants were sampled by cutting the switchgrass close to the soil as possible in a randomly picked area of 0.25 m^2 within the 1 m^2 treatment plots. Plant biomass were placed in pre-labeled paper bags. The paper bags were weighed before biomass samples were put into them, then weighed once again after the fresh biomass samples were placed into the paper bags. The bags were then transported into a drying room to be dried for 72 hours at 80°C[18] in order to record a constant dry weight. Once samples were dried, the bags were weighed once again to calculate weights on an oven-dried basis. All biomass sample dry weights were converted to Mg ha^{-1}.

DNA Extraction and Quantification of Total Bacterial and Fungal Communities

DNA was extracted from homogenized soil samples (0.5 g) within 24 hours of sampling

using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following manufacturers guidelines. A total volume of 100 µL of DNA was obtained and stored at -20 °C until further analysis. The total quantity and purity of DNA was determined using Nanodrop ND-8000 spectrophotometer (Thermo Fisher Scientific Corp.).

Quantitative polymerase chain reaction (qPCR) assay was used to target and enumerate genes for total bacteria (16S rRNA) and total fungal (18S rRNA) communities using primer pairs 338F/518R[19] and FR1/FF390[20].

In both, the total bacteria and total fungal, 20 µL qPCR reaction mixture had 10 µL of Sso Fast™ EvaGreen Supermix (BioRad Laboratories Inc.), 1 µL of forward primer and reverse primer (10 µM), 6 µL of DNase-free water, and 2 µL of 20X diluted template DNA. Polymerase chain reaction (PCR) inhibition tests were conducted according to Mafa-Attoye, *et al.* (2019)[21].

Quantitative PCR conditions were optimized using a CFX96 Touch™ Real-Time PCR Detection System (BioRad Laboratories Inc.). For 16S rRNA: 98 °C (3 min), 40 cycles of dissociation for 98 °C (10 s), annealing of primers for 55 °C (5 s), and extension (30 s) at 72 °C, and 18S rRNA: 95 °C (3 min), 39 cycles of dissociation for 95 °C (15 s), annealing of primers for 50° C (30 s), and extension (45 s) at 72 °C. In both assays, temperature was increased by 0.5 °C from 65 to 95 °C for 5 seconds to analyze melt curves. To enumerate gene copies, standard curves were created using duplicate tenfold dilution series of organism plasmid DNA containing the target genes (organisms in the plasmid 16S and 18S). Each assay also contained two no-template controls which had negligible values.

Penicillium spp. Enumeration and Plant Tissue Sampling

Penicillium-spp. Enumeration was completed by A&L Canada Laboratories Inc. (London, Ontario, Canada). Enumeration was recorded as CFU g^{-1} of dry soil. Soil samples were taken using a sterilized auger (by wiping the auger with alcohol) to a depth of 15 cm for *Penicillium-spp.* enumeration. Collected soil samples were delivered on the same day to the above laboratory for enumeration in order to prevent any loss of microorganisms.

Plant tissue samples were taken in the fall, 2018 and tested for tissue nutrient levels at the Agriculture & Food Laboratories (Guelph, Ontario, Canada). Nutreints that were tested for were total N, P, K, Mg, and Ca.

Statistical Analysis

Statistical analysis was performed using R Core Team version 3.4.0[22] with a Type I Error rate of α=0.05. A one-way analysis of variance (ANOVA) was performed using the *aov* procedure in R and a Tukey's test was performed to determine significance of the treatments for the greenhouse and field experiments.

Results and Discussion

Greenhouse Experiment

The greenhouse experiment had one sampling period, which occurred after 120 days from transplanting, simulating the end of a typical growing season. The biomass yield results are presented in g L^{-1} based on growing media volume and as a percent change in biomass weight compared to the zero-control treatment. The *V. paradoxus* JM63, JumpStart® and inorganic fertilizer treatments all produced biomass yields that were significantly higher than the zero-control (Figure 1). Further, all biofertilizer treatments (*V. paradoxus* JM63 and JumpStart®) had comparable crop yields to that of the inorganic fertilizer treatment (Figure 1). The *V. paradoxus* JM63 had the highest but not statistically different yield of 2.74 (±0.24) g L^{-1}, followed by JumpStart® with a yield of 2.55 (±0.10) g L^{-1}, inorganic fertilizer yield at 2.52 (±0.24) g L^{-1}, and zero control yielded the lowest at 1.34 (±0.09) g L^{-1}.

Significantly higher biomass yields were derived from all treatments when compared to the zero-control treatment. *V. paradoxus* JM63 is an

effective biofertilizer that has been applied previously in *Zea mays* (corn) experiments[23], and it now appears that *V. paradoxus* JM63 can also be used as biofertilizer for switchgrass production. As corn and switchgrass are both C_4 crops, switchgrass seeds too can be inoculated with *V. paradoxus* JM63 prior to planting to derive comparable biomass yields as obtained in fertilized treatment in this study. JumpStart® also provided biomass yield result that was comparable to inorganic fertilizer treatment used in this study. In the literature, there are studies that show yield increases of up to 66% when wheat (*Triticum aestivum* L.) and canola (*Brassica juncea*) were inoculated with JumpStart® [24]. In this study, switchgrass biomass yield increased by 90.2% compared to the zero-control treatment (Table 1), suggesting that JumpStart® can also be an effective biofertilizer for herbaceous biomass crops.

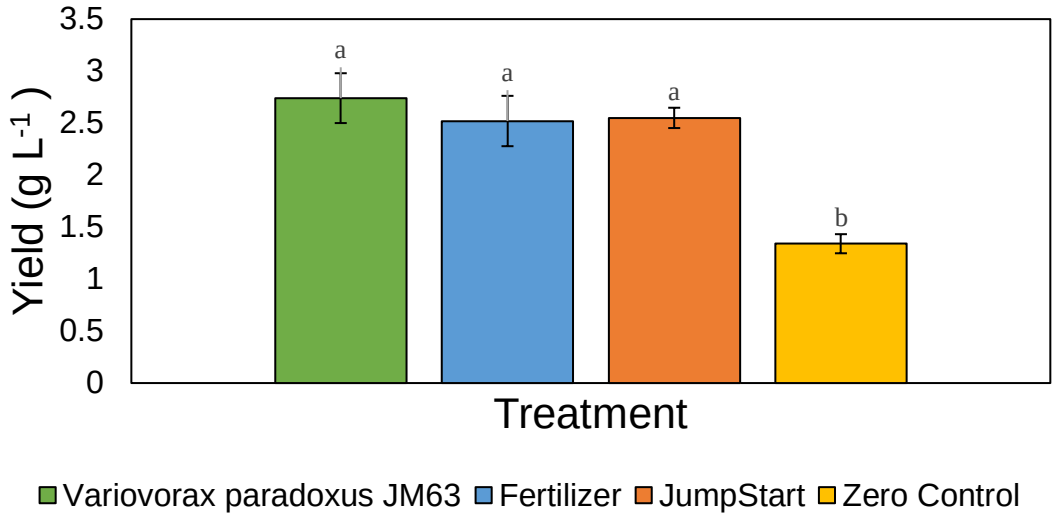

Figure 1: Switchgrass (*Panicum virgatum*) yield (g L^{-1}) as influenced by treatments in the Greenhouse Experiment (n=6). Error bars denote standard error of the mean. Bars with the same letter are not significantly different according to Tukey test (P≥0.05).

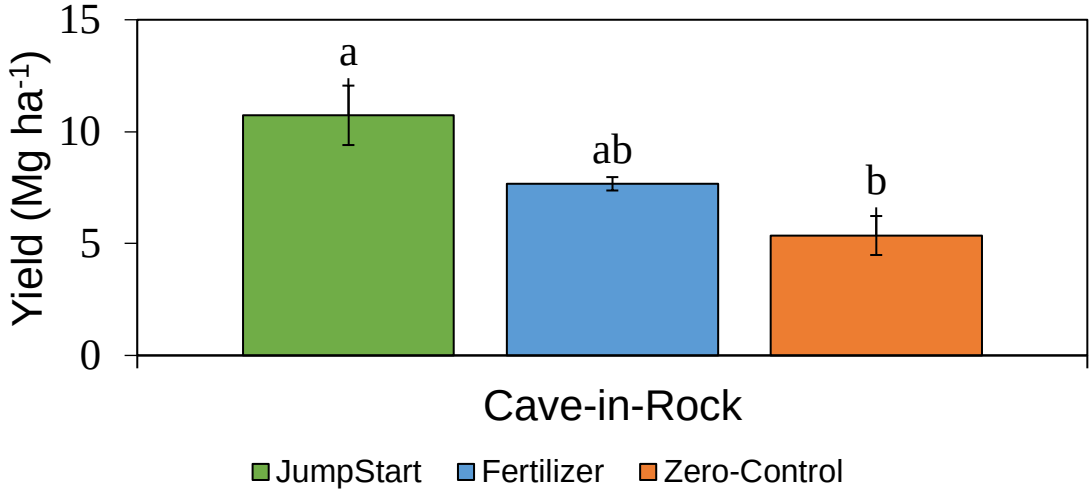

Figure 2: Four-year-old switchgrass (*Panicum virgatum*), cultivar 'Cave-in-Rock' yields (Mg ha^{-1}) as influenced by treatments in a field experiment at the Guelph Turfgrass Institute, Guelph, Ontario, Canada. Error bars denote standard error of the mean (n=4). Error bars labelled with the same letters (a-b) are not significantly different according to Tukey test (P≥0.05).

Table 1: Above ground yield percent change compared to the zero-control from both Greenhouse Experiments (cultivar: 'Cave-in-Rock').

Treatment	Percent Change (%)
Inorganic fertilizer	88.0
Penicillium bilaii (JumpStart®)	90.2
Variovorax paradoxus JM63	104.5
Zero-control	-

Table 2: Soil DNA tested in an autumn sampling for bacteria (16S) and fungi (18S) under JumpStart®, fertilizer, and zero-control.

	Bacteria (16S gene copies /g dry soil)	Fungi (18S gene copies /g dry soil)
JumpStart	1.3×10^{10}	6.8×10^{7}
Fertilizer	1.4×10^{10}	8.3×10^{7}
Zero-Control	1.0×10^{10}	$5.4x\ 10^{7}$

Both DNA (16S and 18S) analyzed by ANOVA (P≥0.05), both were found to be statistically not significant. P-values are as follows: 16S p=0.33 and 18S p=0.16.

Table 3: Plant tissue nutrients (Total N, P, K, Mg, Ca) for Switchgrass (cultivar: 'Cave-in-Rock') from an autumn harvest.

	Total N (ppm)	P (ppm)	K (ppm)	Mg (ppm)	Ca (ppm)
JumpStart	4850 (±330.40)	897.5 (±72.84)	4400 (±467.26)	1000(±81.65)	3325 (±466.15)
Fertilizer	4725 (±631.63)	915 (±167.85)	3750 (±684.96)	1075 (±125.00)	3975 (±981.81)
Zero-Control	4775 (±314.58)	832.5 (±58.51)	3725 (±311.92)	1150 (±165.83)	3750 (±539.29)

All nutrients analyzed by ANOVA (P≥0.05), each were found to be statistically not significant. P-values are as follows: Total N p=0.98, P p=0.86, K p=0.59, Mg p=0.0.34, and Ca p=0.81.

Based on the results from greenhouse experiment, the selected field experiment treatments were inorganic fertilizer and JumpStart® as these treatments provided significantly higher biomass yields compared to the zero-control switchgrass yield. Due to limitations in the laboratory and access to *V. paradoxus* JM63 source, it was not used in field experiment. However, JumpStart® is a commercially available biofertilizer, which the biomass growers can freely access.

Field Experiment

JumpStart® produced significantly higher biomass yields than the zero-control, while biomass yield from the inorganic fertilizer treatment and the zero-control yield were comparable (Figure 2). In the switchgrass variety 'Cave-in-Rock' the JumpStart® treatment had

the highest yield of 10.73 (±1.33) Mg ha^{-1}, the inorganic fertilizer treatment had a yield of 7.67 (±0.30) Mg ha^{-1}, and the zero-control yielded 5.36 (±0.87) Mg ha^{-1}.

In the field experiment conducted in 2018 on research plots established in 2014, JumpStart® performed better with a yield of 10.73 (±1.33) Mg ha^{-1} compared to inorganic fertilizer yield of 7.67 (±0.30) Mg ha^{-1}. The higher yields in the 2014 established crop are comparable to the yields of an established switchgrass crop reported by Marsal *et al* (2016), where unfertilized switchgrass yielded 5.94 (±5.18) Mg ha^{-1} and fertilized switchgrass yielded 14.79 (±3.51) Mg ha^{-1}.

Soil Fungal (18S) DNA and bacteria (16S) DNA were accounted for in the different treatments. JumpStart® and fertilizer treatments had greater amounts of both 18S and 16S DNA compared to the zero-control (Table 2), but it was determined that there is no difference in 18S or 16S DNA (ANOVA $P \geq 0.05$) amounts between treatments.

Enumeration of *Penicillium* spp. in the soil was accounted for in JumpStart® and the zero-control treatments in the field. JumpStart® had a greater amount of *P.* spp. than the zero-control, with 20,033 ± 12,793 CFU g^{-1} of soil compared to <100 ± 12.5 CFU g^{-1} of soil. Greater presence of *P.* spp. in the soil indicates that JumpStart® has successfully inoculated the soil microbiome more so than the zero-control treatment. The fact that the applied JumpStart was still present in the soil after an autumn harvest was interesting. Based on this finding, plant tissue samples were analyzed to determine if more phosphorous was present in the JumpStart® treated switchgrass crop (Table 3) because JumpStart® actively solubilizes phosphorous for plant uptake. There were no statistical differences in phosphorous uptake between the treatments (Table 3). It is believed that plants do translocate their tissue nutrients to the roots as a conservation strategy for next year's growth[25]. In this study, switchgrass was harvested in October 2018 and due to late sampling of the biomass, the switchgrass crop might have translocated the phosphorous to roots and what remained was excess or structural P, thus the low levels of phosphorous found in the above-ground plant tissues.

Conclusion

The objective associated with the studies was to investigate which biofertilizer would be most effective for growing switchgrass in terms of biomass yields when compared to commercially available inorganic fertilizer treatment. Additionally, biofertilizers can have an environmental benefit in the reduction of yearly GHGs from annual fertilization application. As indicated above, inorganic fertilizers all have detrimental environmental implications, when not managed correctly. Therefore, the application of biofertilizers to herbaceous biomass crops can become a novel best management practice that can be recommended to biomass growers. In this context, the results indicate that the known PGPR and JumpStart® could be applied to switchgrass to increase yields that are statistically comparable to inorganic fertilizer application. As *V. paradoxus* JM63 and JumpStart® are not crop specific, and are free-living organisms, they could be beneficial for the soil microbiome as well as to enhance plant growth[6,10]. The enhanced number of *P*. spp. in the soil at the end of the growing season indicate that JumpStart® was able to successfully inoculate into the soil microbiome in order to enhance plant symbiosis. From the results derived from these studies, it appears that biofertilizers can be substituted for inorganic fertilizer use and the fact that JumpStart® is commercially available it could be recommended to Ontario producers to replace/supplement inorganic fertilizer use. However, the presence of microorganisms in the soil for multiple years, in this case, *Penicillium bilaii* (JumpStart), and their influence on long-term biomass yield needs to be verified before firm conclusions or recommendations can be made. This gap needs to be addressed in future studies in Ontario, Canada.

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Abbreviations Greenhouse gas (GHG), Plant growth promoting rhizobacteria (PGPR), Guelph Turfgrass Institute (GTI), Randomized complete block design (RCBD), Colony forming unit (CFU), Megagram (Mg), Quantitative polymerase chain reaction (qPCR), Polymerase chain reaction (PCR), Ribosomal ribonucleic acid (rRNA).

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