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Effects of Irrigation and Nitrogen Fertilization on Seed Yield, Yield Components, and Water Use Efficiency of *Cleistogenes songorica*

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Abstract: Irrigation and nitrogen (N) are two crucial factors affecting perennial grass seed production. To investigate the effects of irrigation and N rate on seed yield (SY), yield components, and water use efficiency (WUE) of *Cleistogenes songorica* (Roshevitz) Ohwi, an ecologically significant perennial grass, a four-year (2016–2019) field trial was conducted in an arid region of northwestern China. Two irrigation regimes (I1 treatment: irrigation at tillering stage; I2 treatment: irrigation at tillering, spikelet initiation, and early flowering stages) and four N rates (0, 60, 120, 180 kg ha^{-1}) were arranged. Increasing amounts of both irrigation and N improved SY, evapotranspiration, WUE, and related yield components like fertile tillers m^{-2} (FTSM) and seeds spikelet^{-1}. Meanwhile, no significant difference was observed between 120 and 180 kg N ha^{-1} treatments for most variables. The highest SY and WUE was obtained with treatment combination of I2 plus 120 kg N ha^{-1} with four-year average values of 507.3 kg ha^{-1} and 1.8 kg ha^{-1} mm^{-1}, respectively. Path coefficient and contribution analysis indicated that FTSM was the most important yield component for SY, with direct path coefficient and contribution coefficient of 0.626 and 0.592. Overall, we recommend I2 treatment (three irrigations) together with 120 kg N ha^{-1} to both increase SY and WUE, especially in arid regions. Future agronomic managements and breeding programs for seed should mainly focus on FTSM. This study will enable grass seed producers, plant breeders, and government program directors to more effectively target higher SY of *C. songorica*.

Keywords: *Cleistogenes songorica*; irrigation; nitrogen application rate; path coefficient analysis; seed yield; seed yield components; water use efficiency

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1. Introduction

Cleistogenes songorica (Roshevitz) Ohwi is a perennial Poaceae grass native to temperate arid, semi-arid, and desert areas of central Eurasia including northern China, Kazakhstan, Kyrgyzstan, Russia, Turkmenistan, Uzbekistan, and many other countries [1]. It is the dominant species in desert grasslands where annual precipitation is around 120 mm, with significant ecological value (Figures S1 and S2) [2]. Because of a strong root system and excellent resistance to stress environments including drought, *C. songorica* has been considered as one of the most promising plant species for desert ecosystem and degraded grassland restoration [3,4]. Recently, a chromosome-level genome was sequenced to illustrate the mechanism underlying the drought tolerance of *C. songorica* [5]. Other than its ecological value, *C. songorica* also attracted more and more agronomic interest due to its good palatability and high nutritional value as forage, which plays a crucial role in developing local animal husbandry and grass-livestock systems [6].

Nevertheless, under native conditions, the seed yield (SY) of *C. songorica* is poor. The insufficient seed cannot meet the increasing demand from ecological restoration, grass-livestock programs, and many other sectors [7]. There is an urgent need to develop feasible methods to achieve a higher SY of *C. songorica*. The grass SY is a complicated quantitative character which is comprehensively affected by the environment, genetic background, and agronomic management [8,9]. As for perennial grass or forage legume—in contrast to grain crops—the progress of SY through plant breeding is scarce since current breeding programs mainly pay attention to vegetative biomass, stress resistance, and feeding quality [10]. As a result, the most practicable method to optimizing SY is to choose an appropriate seed production region and optimize the field agronomic managements used [11].

Water availability and nitrogen (N) nutrient are two principal factors influencing growth and development of perennial grass, which are pivotal in deciding ultimate SY [12]. For perennial grass, water-deficit stress is well-known to cause serious yield loss [13], especially under arid and semi-arid environments [14], and irrigation is needed to avoid high SY penalty. On the other hand, it is noteworthy that perennial grass SY responses to water availability generally varied at different growth stages [12]. Previous research indicates that water deficiency during reproductive periods (such as heading and flowering stage) seems more detrimental for grass seed production because embryo abortion is caused by drought [15,16]. As a result, irrigation application at critical reproductive growth stages may a rational strategy for both SY and water use efficiency (WUE). Huettig et al. [17] found that a single irrigation applied at flowering lead to an average 31% increase for SY and 5% increase for WUE of six tall fescue [*Schedonorus phoenix* (Scop.) Holub] cultivars. We hypothesized that adding water during reproductive period of *C. songorica* may also be beneficial for improving both SY and WUE.

The N is another key factor affecting growth and seed production of perennial grass because it stimulates fertile tiller formation and seed development [18,19]. The significance of N fertilization for grass seed production has been well recorded: Han et al. [20] reported that 190 and 140 kg N ha^{-1} are needed to maximize SY of turf-type and forage-type tall fescue under arid environment of northwestern China. In northeastern China's Songnen Grassland, at least of 120 kg N ha^{-1} is required to improve SY of Chinese sheepgrass (*Leymus chinensis* Trin.) [21]. Perennial ryegrass (*Lolium perenne* L.) seed crops in Oregon of USA need 60 kg N ha^{-1} for maximum SY and seed quality [22]. As for perennial native grass, the knowledge is relatively rare. Previously, only López-García et al. [23] reported the N management practices for plains bristlegrass [*Setaria vulpiseta* (Lam.) Roem. and Schult.] and streambed bristlegrass [*Setaria leucopila* (Scribn. and Merr.) K. Schum.] native to the southern Great Plain of North America, the highest SY was obtained with the N application of 75 to 140 kg ha^{-1}.

The SY of perennial grass is affected directly or indirectly by the contributions of several SY components, such as the number of fertile tiller, number of seed, and seed weight [24]. Each yield component usually has a different level of significance for SY [25]. In such situation, analysis of critical SY components and contributions of each component to SY would gain knowledge of the formation of SY, which would in turn provide insights into selection for higher SY [26]. Path coefficient analysis (PCA) is a powerful tool for determining the contribution of each yield component to SY because this approach separates direct and indirect effects and measures the relative significance of the causal factors involved [24]. The PCA would provide us with an understanding of the nature of the interrelationships among SY and yield components [25] and has been successfully applied in Chinese sheepgrass [21], perennial ryegrass [24], switchgrass (*Panicum virgatum* L.) [27], smooth bromegrass (*Bromus inermis* L.) [25], and many other perennial grasses [15,28,29].

Although research concerning the irrigation and N fertilization for seed crops has been implemented for many perennial grasses, to the best of our knowledge studies on these important factors and their interaction effects for SY and water use characteristics of native grass *C. songorica* remain nonexistent. Besides, the contributions of several yield components to SY are still not very clear. Thus, the objective of this four-year field experiment was to determine the irrigation, N fertilization, and interaction effects on seed production and water use traits of *C. songorica*. We also aimed to analyze and describe the yield components responsible for SY by calculating path and contribution coefficients.

2. Materials and Methods

2.1. Study Site

The field experiments were conducted during four consecutive years (2016–2019) at Minqin Experimental Station of Lanzhou University in Wuwei, Gansu Province, northwestern China (38°44′ N, 103°01′ E; 1307 m above sea level). The study site is located in a typical arid environment with a frost-free period of approximately 160 days. The average annual temperature was 8.8 °C and annual mean precipitation was 120 mm, with 90% of the total precipitation occurring during *C. songorica* growing season (April to September). The soil of the study site is a sandy loam with an average dry bulk density of 1.39 g cm^{-3}. Before planting, the upper 30 cm soil had a pH of 7.68, contained total N of 6.89 g kg^{-1}, organic matter of 12.16 g kg^{-1}, available N of 28.72 mg kg^{-1}, available phosphorus of 17.53 mg kg^{-1}, and available potassium of 113.95 mg kg^{-1}. Before this experiment, silage maize (*Zea mays* L.) was grown at the site for three consecutive years. Monthly precipitation and monthly mean temperature during the growing seasons from 2016 to 2019 were reported in Figure 1.

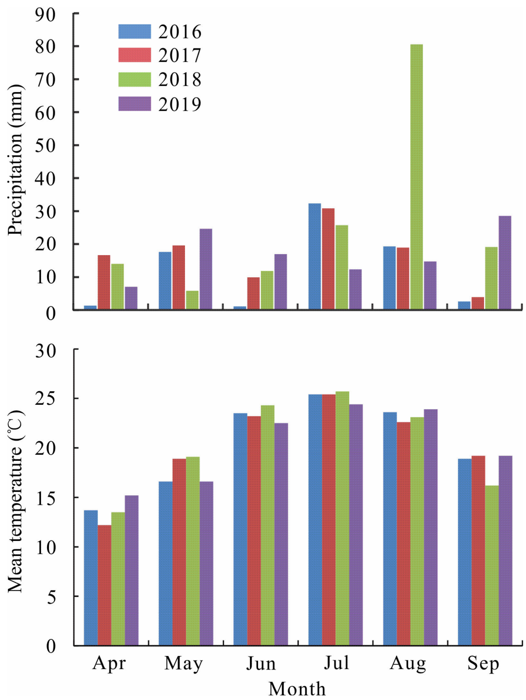

Figure 1. Monthly precipitation and mean temperature at Minqin Experimental Station of Lanzhou University during the growing seasons (April to September) from 2016 to 2019.

2.2. Experimental Design and Plot Maintenance

Treatments were arranged in a split-plot, randomized complete block design with three replications. The main plot treatments were irrigation with 50 mm at tillering stage (I1 treatment) and with 50 mm each at tillering, spikelet initiation, and early flowering stages (I2 treatment). A total of 50 mm and 150 mm water were added in I1 and I2 treatment yearly, respectively. In each irrigation event, water was added through a 63-mm plastic pipe, and a flow meter was used to measure the amount of water supplied. The subplot treatments were four N rates consisting of 0, 60, 120, and 180 kg ha^{-1} in each year, with half of the annual N applied in tillering stage, and the other half applied in spikelet initiation stage. The form of N was urea $[CO(NH_2)_2]$ and the fertilizer was broadcasted over soil and then raked lightly to incorporate with surface soil. The aims of one irrigation event in

I1 treatment were to facilitate the N treatments because the precipitation during tillering (mid-May) was limited (Figure 1), and also ensure the grass survival under arid growth environment. Individual treatment plots were 4 m by 4 m, with 3-m buffering zone between main plots, and 1-m spacing between adjacent plots in main plot. Weeds were removed by hand as needed during each growing season. No visible pests, pathogens, or plant disease were observed during experimental period, thus pesticide was not included in the present experiment.

In our research area, the re-growth of *C. songorica* was beginning in early April. Tillering, spikelet initiation, and early flowering stages occurred in mid-May, mid-July, and early August, respectively. The plots were harvested in late September when seeds matured from 2016 to 2019.

The grass was sown on 20–21 June 2014 by manually planting at 6.5 kg ha^{-1} of seed with 30 cm row spacing. To ensure the successful establishment, diammonium phosphate [$(NH_4)_2HPO_4$] and urea were applied before planting with 150 and 260 kg ha^{-1}, respectively. The 50 mm water was applied to all plots immediately after sowing and also on 1 July, 20 July, and 21 August 2014. The 50 mm water was applied on 11 November 2014 to guarantee winter survival.

2.3. Data Collection

The SY was determined from three, 1-m row segments taken from the center of each plot. Seed samples were dried, threshed, cleaned, weighted, and values converted to SY (kg ha^{-1}) when the seed water content was 8% to 10%. The yield components considered in this study included fertile tillers m^{-2} (FTSM), spikelets fertile tiller^{-1} (SFT), seeds spikelet^{-1} (SS), and thousand seed weight (TSW). Three random 0.5-m row samples in each plot were sampled to measure the FTSM before seed harvest. Then, 50 undamaged fertile tillers and 50 spikelets in each plot were selected to determine the SFT and SS, respectively. Three 1000-seed samples from each plot were counted and weighted for the determination of TSW.

Before *C. songorica* re-growth and after harvesting in each growing season, soil water content (SWC) to a depth of 200 cm was measured for calculating soil water storage (SWS) in the soil profiles. One soil core (42 mm diameter) in each plot between two central rows were manually sampled at 20-cm intervals, after their wet weight was weighted, the soil samples were dried at 105 °C to a constant weight, the SWC was calculated on the basis of soil dry weight. The SWS was calculated according to the equation [30]:

$$SWS = \sum h_i \times p_i \times b_i \ / \ 10 \tag{1}$$

where h_i is the soil thickness (cm), p_i is the soil bulk density (g cm^{-3}), b_i is SWC of each layer, and i = 20, 40, 60 . . . , 200. Evapotranspiration (ET, mm) was calculated using water balance formula [31]:

$$ET = P + I + SWS_0 - SWS_1 \tag{2}$$

where P is precipitation during growing seasons (mm), I is irrigation amount (mm), SWS_0 and SWS_1 are SWS (mm) before re-growth and after harvesting. WUE (kg ha^{-1} mm^{-1}) was calculated using equation [32]:

$$WUE = SY \ / \ ET \tag{3}$$

2.4. Statistical Analysis

All statistical analysis was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Data of SY and its components, ET, and WUE were subjected to analysis of variance (ANOVA). In the combined analysis, year, irrigation, and N treatment were treated as the fixed factors while block was considered as random factor. Significant differences between treatments were compared by Duncan's multiple comparisons test at $p < 0.05$ level. All determinations reported were the means of three replications. The correlation and path coefficients were calculated to estimate the relative significance of each yield component to SY (n = 96), the contribution coefficient was defined to be the correlation coefficient of the SY and yield components multiplied by corresponding path coefficient, as described by Wang et al. [21].

3. Results

3.1. Weather Conditions

The monthly precipitation was quite variable during the four experimental years (Figure 1). The total precipitation in the growing seasons from 2016 to 2019 was 75.4, 100.9, 158.1, and 105.2 mm, respectively. During anthesis and seed set period (August and September) there was obviously higher precipitation in 2018 (100.0 mm) than that in 2016 (22.3 mm), 2017 (23.2 mm), and 2019 (43.6 mm). The monthly mean temperature showed less variation between four years and followed the typical pattern of temperate continental climate (Figure 1).

3.2. Seed Yield

The ANOVA indicated that SY was significantly influenced by all factors ($p < 0.01$) and by the irrigation × N interaction ($p < 0.05$) (Table 1).

Table 1. Analysis of variance for year, block, irrigation, nitrogen application, and their interaction effects on seed yield, yield components, evapotranspiration, and water use efficiency of *Cleistogenes songorica*.

Items	Year (Y)	Block	Irrigation (I)	Nitrogen (N)	Y × I	Y × N	I × N	Y × I × N
Degree of freedom	3	2	1	3	3	9	3	9
Seed yield	***	ns	**	***	ns	ns	*	ns
Fertile tillers m^{-2}	***	ns	**	***	ns	***	***	ns
Spikelets fertile tiller^{-1}	***	ns	ns	*	ns	ns	ns	ns
Seeds spikelet^{-1}	***	ns	**	***	***	***	*	ns
Thousand seed weight	***	ns	*	***	***	ns	ns	ns
Evapotranspiration	***	ns	***	***	***	ns	ns	ns
Water use efficiency	***	ns	*	***	ns	ns	ns	ns

Note: * Indicates significance at $p < 0.05$ level; ** Indicates significance at $p < 0.01$ level; *** Indicates significance at $p < 0.001$ level; ns, not significant at $p < 0.05$ level.

The irrigation treatment affected SY sharply in both years. The SY with I2 treatment increased compared with I1 treatment by 41.2% in 2016, 81.6% in 2017, 38.6% in 2018, and 158.8% in 2019. In both years and irrigation treatments, the SY presented increasing responses with the increasing N doses up to 120 kg ha^{-1} and then decreased or stabilized when N rate increased to 180 kg ha^{-1}. There was no significant difference for SY between 120 and 180 kg N ha^{-1} (Figure 2).

Significant irrigation × N effect was observed, the highest SYs were achieved with the treatment combination of I2 + 120 kg N ha^{-1} and I2 + 180 kg N ha^{-1}, with the average SY over four years of 507.3 and 520.4 kg ha^{-1}, respectively (Figure 2).

Except for treatments, yearly variation for SY was also evident, across all treatments, the average SY from 2016 to 2019 was 398.8, 342.2, 443.2, and 196.2 kg ha^{-1}, respectively (Figure 2).

In contrast to SY, seed quality was not significantly affected by irrigation and N fertilization treatments, similar final germination percentage (FGP), germination index, and mean germination time was observed for seeds harvested from different treatments. The FGP exceeded 90% in all treatments (data not shown).

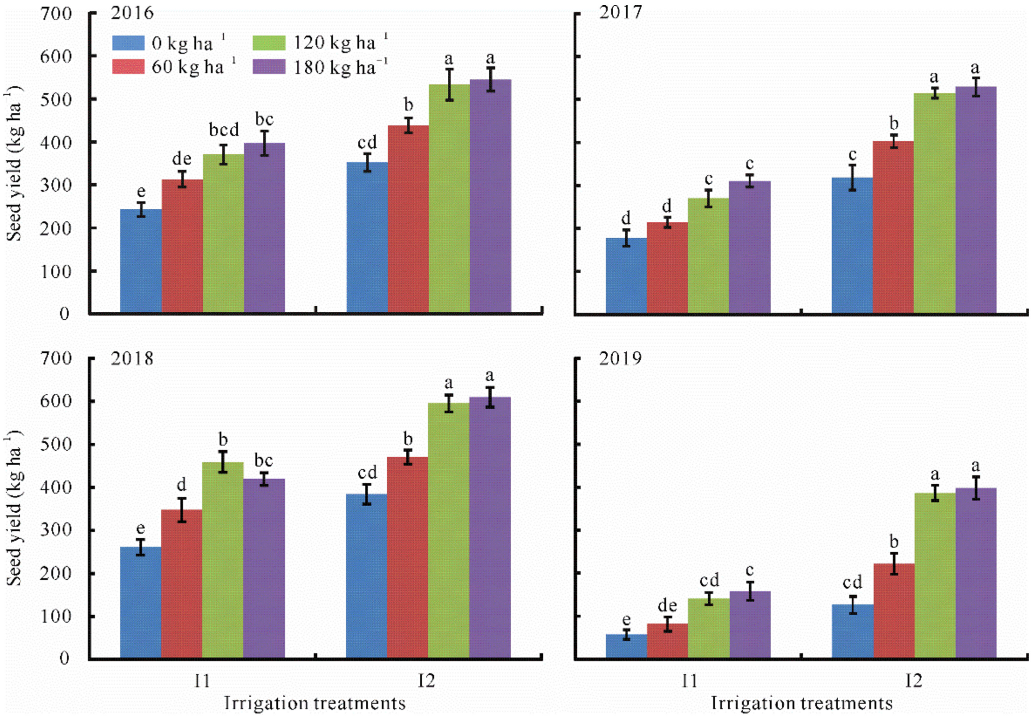

Figure 2. Effects of different irrigation and nitrogen rate treatments on *Cleistogenes songorica* seed yield from 2016 to 2019. Bars represent the standard errors (n = 3). Different lowercase letters above bars within a year indicate significant difference at $p < 0.05$ level. I1: watering at tillering stage, a total of 50 mm water was added in each year; I2: watering at tillering, spikelet initiation, and early flowering stages, respectively, a total of 150 mm water was added in each year.

3.3. Seed Yield Components

All yield components were significantly affected by year, irrigation, and N treatment, except for irrigation effect which was not significant for SFT. The FTSM was significantly influenced by year × N and irrigation × N interaction. The SS was significantly influenced by year × irrigation, year × N, and irrigation × N interaction. In addition, there was a significant effect of year × irrigation interaction for TSW. The three-way interaction had no impact on all tested yield components (Table 1).

Both yield components varied with irrigation and N treatments, except for SFT, which was relatively stable between treatments, there was only significantly difference between treatments I1 + 0 kg N ha^{-1} and treatments I2 + 60, 120, and 180 kg N ha^{-1} in 2018 (Table 2). Compared with I1 treatment, the I2 treatment significantly improved FTSM and SS by 54.4% and 22.5% across four years and N rate treatments. Furthermore, when the means of FTSM and SS were compared between four N rates, we found that increasing N dose promoted positive effects, but no significant difference was recorded between 120 and 180 kg N ha^{-1} treatments (except for SS in 2017, I1 treatment). Similar to SY, the highest values were observed in the treatment combinations of I2 + 120 or 180 kg N ha^{-1} (Table 2).

The TSW tended to increase with increasing irrigation and N dose, although the responses were neither consistent nor were they always statistically significantly. The I2 treatment increased the TSW by 0.001–0.006 g relative to I1 treatment, and fertilized plots showed 0.001–0.005 g greater TSW over zero N plots during four years (Table 2).

Table 2. Effects of different irrigation and N rate treatments on *Cleistogenes songorica* seed yield components from 2016 to 2019.

Year	Irrigation Treatments	N Rates (kg ha^{-1})	Fertile Tillers m^{-2}	Spikelets Fertile Tiller^{-1}	Seeds Spikelet^{-1}	Thousand Seed Weight (g)
2016	I1	0	633.1e	9.01a	26.1f	0.225bc
		60	881.2d	8.91a	28.4e	0.226abc
		120	1266.2b	8.84a	31.5c	0.227a
		180	1252.2b	8.81a	31.9bc	0.227ab
	I2	0	985.5c	8.99a	29.7d	0.225c
		60	1354.9b	8.93a	33.0b	0.227abc
		120	1800.6a	8.87a	37.7a	0.228a
		180	1863.6a	8.83a	37.4a	0.227a
2017	I1	0	627.7e	8.71a	21.5e	0.238e
		60	910.0d	8.65a	25.5d	0.241cd
		120	1109.1c	8.59a	30.1c	0.242cd
		180	1168.2c	8.61a	32.8b	0.242cd
	I2	0	908.4d	8.66a	27.8d	0.241d
		60	1268.6b	8.57a	30.1c	0.243bc
		120	1662.9a	8.51a	37.3a	0.243b
		180	1700.2a	8.52a	37.3a	0.245a
2018	I1	0	752.1e	8.47a	25.1d	0.237d
		60	1098.2d	8.35ab	29.1c	0.241abc
		120	1582.8bc	8.21ab	35.1b	0.240bc
		180	1666.8b	8.29ab	34.8b	0.242ab
	I2	0	1128.6d	8.32ab	27.2cd	0.239c
		60	1527.6c	8.15b	33.1b	0.241abc
		120	2211.2a	8.10b	40.8a	0.243a
		180	2149.8a	8.11b	40.1a	0.242ab
2019	I1	0	367.9e	9.18a	17.1e	0.215d
		60	512.6d	9.15a	18.8d	0.216d
		120	655.6c	9.15a	24.4c	0.218cd
		180	745.9c	9.21a	24.5c	0.218cd
	I2	0	650.2c	9.08a	24.5c	0.220bc
		60	861.0b	9.07a	28.6b	0.222ab
		120	1277.1a	9.06a	33.2a	0.224a
		180	1370.5a	9.01a	33.7a	0.224a

Note: Different lowercase letters within a column and year indicate significant difference at $p < 0.05$ level. I1: watering at tillering stage, a total of 50 mm water was added in each year; I2: watering at tillering, spikelet initiation, and early flowering stages, respectively, a total of 150 mm water was added in each year.

3.4. Contributions of Seed Yield Components to Seed Yield

All yield components are significantly correlated with SY ($p < 0.001$), the FTSM, SS, and TSW are positively correlated, while SFT is negatively correlated. The largest correlation coefficient was FTSM on SY (0.945) (Table 3).

Table 3. Correlation coefficients, path coefficients and contributions of yield components to the seed yield of *Cleistogenes songorica* ($n = 96$).

Seed Yield Components	Correlation Coefficient with SY	Direct Path Coefficients	Indirect Path Coefficients				Contributions to SY
			FTSM	SFT	SS	TSW	
FTSM	0.945 ***	0.626		−0.020	0.286	0.053	0.592
SFT	−0.577 ***	0.033	−0.377		−0.149	−0.084	−0.019
SS	0.926 ***	0.301	0.595	−0.016		0.047	0.279
TSW	0.546 ***	0.099	0.333	−0.028	0.142		0.054

Note: *** Indicates significance at $p < 0.001$ level. FTSM: fertile tillers m^{-2}; SFT: spikelets fertile tiller^{-1}; SS: seeds spikelet^{-1}; TSW: thousand seed weight; SY: seed yield. A yield component contribution to SY was defined as the correlation coefficient between the SY and the yield component multiplied by the corresponding direct path coefficient.

The PCA indicated that FTSM had the strongest direct effect on SY with direct path coefficient of 0.626, followed by SS (0.301), TSW (0.099), and SFT (0.033). Additionally, the SS and TSW also indirectly influenced SY via FTSM, and the corresponding indirect path coefficient is 0.595 and 0.333 (Table 3).

Likewise, the analysis of contribution revealed that FTSM contributed to SY mostly, with the contribution coefficient of 0.592 (Table 3).

3.5. Evapotranspiration and Water Use Efficiency

The ET was significantly influenced by year, irrigation, and N rate treatment (Table 1), ranged from 225.6 to 302.0 mm, 193.1 to 267.8 mm, 244.0 to 307.9 mm, and 137.6 to 264.3 mm in 2016, 2017, 2018, and 2019 growing seasons, respectively (Table 4). ET in I1 treatment was significantly lower than in I2 treatment during four years, but ET only significantly increased as N amount increased in 2016 and 2019. In 2017 and 2018, though an increased trend was observed with the increase of N amount, ET did not exhibit statistically significant difference between four N rate treatments (Table 4).

Table 4. Effects of different irrigation and N rate treatments on evapotranspiration and water use efficiency of *Cleistogenes songorica* seed crops from 2016 to 2019.

Year	Irrigation Treatments	N Rates (kg ha^{-1})	Soil Water Storage Before Re-Growth (mm)	Soil Water Storage After Harvesting (mm)	Evapotranspiration (mm)	Water Use Efficiency (kg ha^{-1} mm^{-1})
2016	I1	0	435.9	335.7	225.6 ^{e}	1.1 ^{c}
		60	436.2	315.0	246.6 ^{d}	1.3 ^{bc}
		120	430.6	302.2	253.8 ^{cd}	1.5 ^{b}
		180	430.0	296.5	258.9 ^{c}	1.5 ^{ab}
	I2	0	437.2	386.2	276.5 ^{b}	1.3 ^{bc}
		60	435.4	373.8	287.0 ^{b}	1.5 ^{ab}
		120	437.3	363.8	298.8 ^{a}	1.8 ^{a}
		180	434.3	357.7	302.0 ^{a}	1.8 ^{a}
2017	I1	0	341.7	299.5	193.1 ^{b}	0.9 ^{e}
		60	323.7	278.4	196.2 ^{b}	1.1 ^{de}
		120	313.4	261.2	203.1 ^{b}	1.3 ^{cd}
		180	312.6	255.3	208.2 ^{b}	1.5 ^{bc}
	I2	0	372.9	377.6	246.3 ^{a}	1.3 ^{cd}
		60	366.5	365.1	252.3 ^{a}	1.6 ^{b}
		120	354.3	341.1	264.1 ^{a}	1.9 ^{a}
		180	350.9	334.0	267.8 ^{a}	2.0 ^{a}
2018	I1	0	284.2	248.3	244.0 ^{b}	1.1 ^{e}
		60	286.7	247.3	247.5 ^{b}	1.4 ^{cd}
		120	278.1	234.6	251.6 ^{b}	1.8 ^{ab}
		180	274.5	224.4	258.1 ^{b}	1.6 ^{bc}
	I2	0	326.7	340.9	293.9 ^{a}	1.3 ^{de}
		60	331.2	343.8	295.6 ^{a}	1.6 ^{bc}
		120	322.8	329.1	301.7 ^{a}	2.0 ^{a}
		180	315.4	315.5	307.9 ^{a}	2.0 ^{a}
2019	I1	0	245.4	263.0	137.6 ^{g}	0.4 $_{d}$
		60	237.3	241.7	150.8 ^{f}	0.5 $_{d}$
		120	228.8	217.1	166.8 ^{e}	0.8 ^{bc}
		180	223.9	201.7	177.4 ^{d}	0.9 ^{b}
	I2	0	315.9	348.2	222.9 ^{c}	0.6 ^{cd}
		60	323.5	338.8	240.0 ^{b}	0.9 ^{b}
		120	313.8	320.2	248.9 ^{b}	1.6 ^{a}
		180	303.4	294.3	264.3 ^{a}	1.5 ^{a}

Note: Different lowercase letters within a column and year indicate significant difference at $p < 0.05$ level. I1: watering at tillering stage, a total of 50 mm water was added in each year; I2: watering at tillering, spikelet initiation, and early flowering stages, respectively, a total of 150 mm water was added in each year. Evapotranspiration = P + I + SWS_0 − SWS_1, where P is precipitation in growing seasons, I is total irrigation, SWS_0 and SWS_1 are soil water storage before re-growth and after harvesting. Precipitation in growing seasons from 2016 to 2019 was 75.4, 100.9, 158.1, and 105.2 mm, respectively.

The WUE was consistently higher in I2 treatment than that in I1 treatment, with the former resulting in 19.7%, 40.6%, 15.6%, and 68.9% greater WUE than the latter from 2016 to 2019, respectively. Among four N treatments, WUE was highest in 120 and 180 kg ha^{-1} treatment and lowest in zero N treatment during four years. The treatment combination of I2 + 120 kg N ha^{-1} and I2 + 180 kg N ha^{-1} consistently had the largest WUE with an average of 1.8 kg ha^{-1} mm^{-1} over four years (Table 4).

Utilizing the observed results over four-year period, we explored the relationship between ET and SY (Figure 3). The relationship was described by a linear regression (r^2 = 0.81; $p < 0.001$), which indicated a linear increase of SY with a unit increase of ET. The relationship predicated an SY increase of 3.2 kg ha^{-1} for each millimeter of ET above the threshold value (137.6 mm) required to produce the minimum SY.

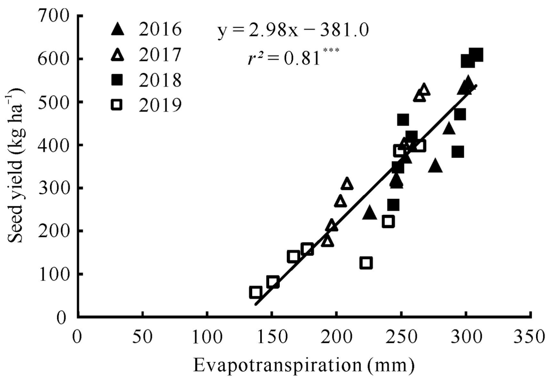

Figure 3. Relationship between evapotranspiration and seed yield of *Cleistogenes songorica* over four-year period. (Note: *** Indicates significance at $p < 0.001$ level).

4. Discussion

The perennial grass SY determines the economic viability of seed growers, and water availability is a key factor influencing SY, especially under arid and semi-arid environments [33]. It has been reported that grass seed crops are very sensitive to water-deficit stress during reproductive periods like anthesis and seed setting stages, thus strategic irrigation to support these critical stages is beneficial for improving SY [13]. In accordance with our hypothesis, the *C. songorica* SY was consistently improved by I2 treatment, despite the yearly precipitation in four growing seasons varying largely (Figure 2). Comparing with I1 treatment, two extra irrigation events during critical reproductive growth period were implemented in I2 treatment, which reduced the probability of water-deficit risk during flowering and subsequent seed filling stage, and ultimately resulted in much higher SY. This result parallels findings of Chastain et al. [34], who found the 16.6% increase of perennial ryegrass SY by a single irrigation at anthesis. However, our finding is in disagreement with a previous experiment conducted in Brazil reporting that irrigation had little influence on SY of signal grass (*Urochloa decumbens* Webster), under humid Brazil condition, water is not considered as a limiting factor [35].

N is an essential macro-element known to influence plant growth and seed development, for N positively participates in numerous plant metabolic processes including photosynthesis and acts as an important constituent of amino acids, chlorophyll, and nucleic acids, etc. [36]. Additionally, some researchers have suggested that N is involved in expression of some flowering genes, and on-off cycles in the gene expression levels was positively correlated with the N fertilization levels, thus in turn N fertilization can effectively increase SY [37]. Results from numerous field trials for perennial grass seed production showed that SY strongly responds to N fertilization; an appropriate N level generally has a positive impact on SY but after which yields are stagnant or reduced [20,23,35,38,39]. Similarly, current experiment showed that, under both irrigation conditions, the SY of *C. songorica* increased with increasing N level but did not differ significantly between 120 and 180 kg N ha^{-1} treatment, which is consistent with earlier reports. Under our growing environment, the N application at a rate of 120 kg ha^{-1} appeared adequate for optimizing *C. songorica* SY, previous reports indicated that recommended N rates for grass seed production was species specific, and also depended on cultivars, environments, and field management [12,13,18,20,23,38–40].

Even though our trial was carried out with irrigation treatments, fluctuation of rainfall between years may play an important role in explaining yearly variation of SY. The obvious decrease of SY in 2019 may be mainly due to less precipitation in July and August. This result is in line with the findings of Wang et al. [15], who demonstrated that scanty precipitation during anthesis was detrimental for seed production of Russian wildrye (*Psathyrostachys juncea* Nevski). Moreover, the increased grass stand age may also be partly responsible for the conspicuous drop in *C. songorica* SY in 2019, as previously reported for Kentucky bluegrass (*Poa pratensis* L.) and intermediate wheatgrass [*Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey] [40–42].

Among four tested yield components, the FTSM and SS were more responsive to irrigation and N fertilization treatments, while SFT and TSW were relatively constant. Typically, FTSM and SS were increased by I2 treatment and increasing N dose (Table 2). Gislum and Griffith [19] had concluded the fundamental function of N fertilization in development of perennial ryegrass fertile tiller. In the near research location with our study, accordant result was also reported by Han et al. [20] in which the fertile tillers number of tall fescues were increased with optimum N level. The better soil water availability in I2 treatment may also be beneficial for stimulating more vegetative tillers transfers to fertile tillers, and also ensure seed development to increase seed number. In addition, the water deficiency during flowering and beginning of seed fill may also cause fertile tiller death and seed abortion [13]. Similarly, a previous report in creeping red fescue (*Festuca rubra* L. var. *rubra*) indicated fertile tiller number was increased by extra irrigation and is in line with our results [12]. Besides, studies in tall fescue and perennial ryegrass had similar results, in which seed number was significantly improved by irrigation at anthesis [17,34].

The spikelet number and seed weight were generally considered as genetically manipulated traits and seldom influenced by agronomic treatments [43,44]. For example, Han et al. [20] found that N application had no impact on tall fescue SFT and was similar with our results in *C. songorica*. The TSW increased somewhat by irrigation and N fertilization treatments (Table 2), and study in timothy grass (*Phleum pratense* L.) had consistent finding [45]. The improvement of seed weight may be mainly due to elevated assimilate uptake, storage, and transfer to seed [14], and also can be attributed to longer seed fill period resulting from supplemental irrigation at reproductive stages [34]. On the contrary, it is worth noting that changes of seed weight are often slight in the whole [46].

The results from PCA and contribution analysis revealed that FTSM had a considerable direct effect on SY, and also contributed most to SY, while other yield components were less important (Table 3). Fertile tiller is the basic biological unit in the formation of SY [25,47], and we found it changed with the yield change correspondingly, which accounted most for the variation of yield. Present research had similar results with those of several studies showing that fertile tiller number was the most significant yield component for SY in Russian wildrye [15], smooth bromegrass [43], creeping red fescue [48], and perennial ryegrass [49]. Nevertheless, our results differ from reports by Wang et al. [50], in which SFT was the most critical yield component for Siberian wildrye (*Elymus sibiricus* L.) SY. This might be because Siberian wildrye SFT was tremendously improved by phosphorus fertilization treatments and needs further research [50]. In the present experiment, SFT had negative indirect influences on SY via FTSM, SS, and TSW, which revealed the trade-off between these components.

In arid and semi-arid regions, water use traits are important for sustainable agricultural production [51]. ET is the total water consumption of agricultural system and is comprehensively affected by agronomic practices, weather conditions, and many factors; therefore, understanding the ET would help improve irrigation management and WUE [52]. Previous reports regarding ET of perennial grass seed crops were limited, but increasing the amount of water and N applied has been widely demonstrated to increase ET of crops including oilseed rape (*Brassica napus* L.), maize, and wheat (*Triticum aestivum* L.) [53–55]. This is because, under sufficient water and N applied situation, soil evaporation increased, meanwhile, larger leaf area and plant biomass resulted from high rate of water and N fertilization, which increased plant transpiration, thereby jointly increasing ET [52,55]. This was also confirmed here for *C. songorica*. In the present study, we found that SY is positively correlated with ET (Figure 3); studies for several crops also got similar results which is not unusual [56–58].

WUE of plants can be influenced by application of irrigation and N fertilization, we found that I2 + 120 kg N ha^{-1} treatment consistently had the highest WUE in four years (Table 4). Our results differ somewhat from previous study for perennial ryegrass, in which single irrigation at anthesis reduced WUE. This may be due to the difference of effective rooting depth between different grasses, and also attributed to higher perennial ryegrass water use in irrigated plots (363 mm) [34]. However, our result is in agreement with the finding of Cookson et al. [59], who suggested that higher rate of N produces greater perennial ryegrass WUE than lower rates.

5. Conclusions

Based on findings of our four-year field trial, three irrigation events during growing season (tillering, spikelet initiation, and early flowering stage) together with an N rate of 120 kg ha^{-1} resulted in increased *C. songorica* SY, WUE, and related yield components including FTSM and SS. Average SY and WUE over four years under this treatment was 507.3 kg ha^{-1} and 1.8 kg ha^{-1} mm^{-1}, respectively. The SY was positively correlated with ET. With the highest direct path coefficient and contribution coefficient, the FTSM was the most pivotal yield component for SY. In conclusion, the irrigation at critical reproductive stages and optimum N application rate were the important aspects for successful seed production. We recommend the treatment combination of I2 + 120 kg N ha^{-1} for *C. songorica* seed production under arid environment of northwestern China, future agronomic managements and breeding objectives for seed should pay attention to FTSM to further increase SY.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/3/466/s1. Figure S1: A picture of *Cleistogenes songorica* plant. Figure S2: A picture of *Cleistogenes songorica* population in desert grassland.

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