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Table: 2, Figs : 3

Effects of mixed sowing of tall fescue (*Festuca arundinacaea* Schreb.) varieties *Millennium 2* and *Arid 3* on growth of lawns

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ABSTRACT

Allelopathic effect can lead to continuous cropping problem in plants. Mixed sowing of different species/varieties may overcome this problem. Two turfgrass (*Festuca arundinacea* Schreb.) varieties *Millennium2* and *Arid3* were studied for their growth potential in mixture and effects on growth of lawns. The results showed that the mixed sowing proportion had significant impact on the growth of turfgrass. The main variety *0* showed improved physiological indices and better growth in mixture ratio of 4:1. The variety *Millennium2* was more sensitive to mixture than *Arid3*. GC-MS analysis showed that the root aqueous extracts of *Millennium2* and *Arid3* contained 15 and 22 allelopathic compounds, respectively, and the content of 1,1-diethoxy-3-methylbutane was highest. Both varieties shared 9-chemicals (1,1-Diethoxybutane, 2-methylpentanoyl 2-methylpentanoate, 1,1-diethoxy-3-methylbutane, octamethylcyclotetrasiloxan, 6-ethyl-2-methyloctane, 5-methylundecane, 4,6-dimethyldodecane, henicosane and icosane), while *Arid3* and *Millennium2* individually contained 13 and 6 unique compounds, respectively. Thus, the selection of compatible species/varieties and their suitable proportion is very important for ideal mixture to improve the appearance of lawn and ecological effects.

Key words: Allelopathy, allelopathic compounds, autotoxicity, continuous cropping problem, *Festuca arundinacea*, GC-MS, lawn, mixed sowing, turfgrass.

INTRODUCTION

The cultivation of same cultivar year by year in the same lawn declines its growth vigour, yield, quality, and resistance to biotic and abiotic stresses. This continuous cropping problem exist widely in lawns, it is caused by the allelopathic autotoxic effects of decomposed tall fescue litter (15). It considerably inhibits its own seed germination and seedling growth and the inhibitory effects increases with the quantity of mass added to lawns. The leaf spot is main diseases of *Festuca,* caused by *Bipolaris sorokiniana*, it could be controlled (20 %) by mixed sowing with *Lolium perenne* and *Poa pratensis* than monoculture of turf (9). The continuous cropping problem is the effect of allelochemicals in soil.

The phenolic acids secreted by cucumber roots decreased the transpiration and photosynthesis of cucumber plants and thereby inhibits their growth (22). Nevertheless, each crop specie or variety produces specific allelopathic chemicals, which exerts different allelopathic effects on others plants (1,5). However, the allelopathic effects of aqueous

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extracts of *Caragana korshinskii* leaves, improved to the seed germination and seedling growth of *Agropyron cristatum* plants at low concentrations but significantly inhibited at high concentrations (23). Likewise, the aqueous extracts of Onion and Cumin, increased the cotton seedlings fresh weight and root activity, at low concentration, but were inhibitory at high concentration (14). Effects of aqueous extracts of aboveground part of bermudagrasses on 5-acceptor grasses were investigated (2). The results showed that the germination and seedling growth of the acceptors, especially *Lolium multiflorum*, *Digitaria sanguinalis* and *Eleusine indica,* were inhibited. Liang (11) assayed the effects of root exudates of '*Baoding*' *Bermudagrass* on seed germination, biomass and physiological processes of two cultivars of *F. arundinacea*. The cultivar *Justice* was more sensitive, while *Greenlabel* was insensitive but its roots were more sensitive. Obviously, the allelopathic effects were specific to specie, variety, tissue and concentration. This would be helpful to alleviate the continuous cropping problem to a certain extent.

Figure 1. The tall fescue variety *Arid3* seeds in above and plants in bottom Photographs

Festuca arundinacea is common cold season lawn grass specie in world (Fig. 1). Its continuous monoculture results in the grass decline. This study aimed to (i). Explore the effects of mixed sowing of 2-tall fescue varieties on their physiological indices to determine the potential in practice application, (ii). Identify the chemical compounds of test varieties and (iii). Find the possible mechanism of effects in mixed sowing. The results may help in overcoming the continuous cropping problems of plants.

MATERIALS AND METHODS

This study was conducted from September 2019 to September 2020 in Beijing Forestry University, Beijing (Longitude, 116º23′17″N; latitude, 39º54′27″E; Altitude, 60 m; Weather conditions: temperate monsoon climate; Annual precipitation: 448 mm).

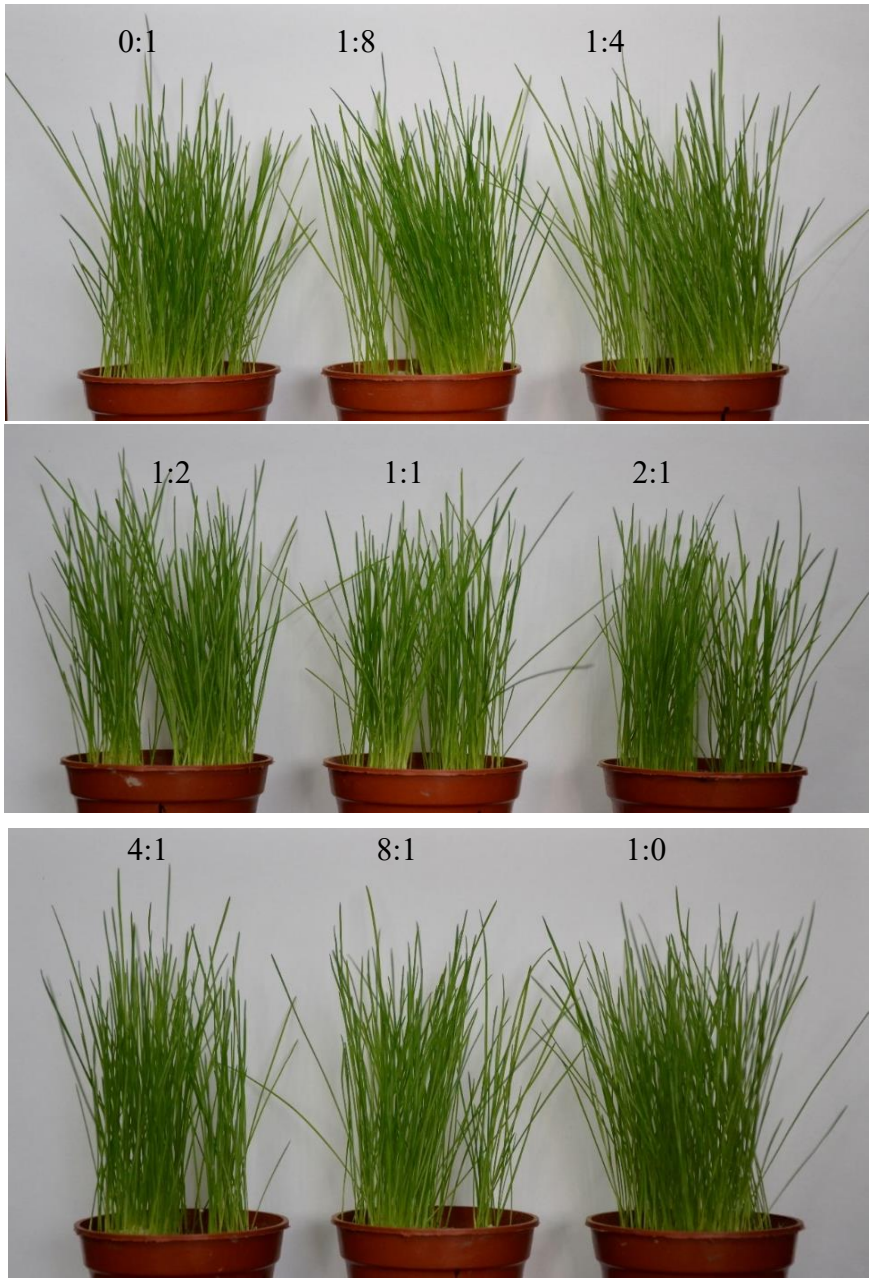

Figure 2. Mixed sowing of *Millennium2* and *Arid3* in various proportion 0:1, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1 and 1:0

1. Pot culture

The seeds of *Festuca arundinacea Schreb. Millennium2* and *Arid3* were purchased from Top Green Company, Beijing. These were disinfected with 10 % of disinfectant 84 (Beijing Longan Medical Technology Development Co. Ltd) for 5 min and washed twice with distilled water. 1.5 g sterilized seeds of both grass varieties were mixed and sown in pots (7 cm x 7 cm x 6 cm) (Fig. 2) in proportion of 0:1, 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1, 1:0 *(Millennium2*: *Arid3*), respectively. Pots were filled with vermiculite and placed in growth chamber (2000 lux light intensity, 16/8 h light/dark cycle, 25 °C). Irrigation with water was done every 3 days and with Hoagland solution once a week (8). After 15 days, leaf and root tissues were collected for physiological indices and gas chromatography-mass spectrometry (GC-MS) analysis.

2. Bioassays

(i). GC-MS analysis

The roots of 15 days old turf grass plants were harvested, cleaned with water and dried in oven (80 ^{0}C) overnight. The samples were powdered with a pulverizer. The 10 ml distilled water was added to 1 g powdered mass to get concentration of 100 mg·ml^{-1}, and shaken in the incubator (28 ^{0}C) for 48 h. The extracts were filtered with gauze and 0.45 µm microporous filter. Five ml extract filtrate was mixed with 2 ml ethyl acetate. Its 1 µl was analysed by GCMS-Qp2010 Ultra GC-MS (manufactured by Shimadzu Corporation). The GC conditions were as under: inlet temperature 250 ^{0}C, helium (99.99 %) carrier gas flow at constant 1ml·min^{-1}, injector split ratio 10:1. The temperature program for GC started at 50 ^{0}C and increased at the rate of 15 °C·min-1 to 180 ^{0}C for 5 min and then at 10 C ·min^{-1} until it reached 250 ^{0}C for 15 min. MS analysis was conducted in positive electron ionization mode. The source temperature was held at 230 ^{0}C, with an electron ionization potential of 70 eV and ions were scanned over the molecular weight range of 33-400 atomic mass units. Each metabolite was identified with the standard mass spectrum database of NIST 11.lib and NIST11s.lib and the relative content of each component was counted by area normalization (10).

(ii). Physiological index evaluation

A. Relative electrolyte leakage (REL): It was measured as per the reference (24). 0.1g fresh leaves were cut into 0.5 cm pieces, soaked in 30 ml deionized water and shaken overnight. The electrical conductivity of the solution was measured as R1. The solution was then boiled for 10 min and shaken overnight again. The electrical conductivity was measured as R2. REL was calculated using the formula of R1/R2.

B. Malondialdehyde (MDA) content: It was measured as per the reference (24). 0.1 g fresh leaves were ground in 1 ml trichloroacetic acid, then centrifuged at 12000 r·min^{-1} for 10 min. The supernatant was mixed with 0.6 % thiobarbituric acid in equal volume and kept in boiling water bath for 15 min. After centrifugation at 12000 r·min^{-1} for 10 min, the supernatant was used to determine the absorbance value at wavelengths of 450, 532, and 600 nm. MDA content (µmol·g^{-1}) was calculated by

following formula:

$$[6.459 \text{ X } (OD_{532} - OD_{600}) - 0.56 \times OD_{450}] \times V/W$$

Where, V: Extraction volume (ml); W: Fresh sample weight (g)

C. Soluble sugar content (SSC): It was measured as per the reference (19). 0.1 g fresh leaves were added in 2 ml deionized water and boiled for 20 min. After centrifugation at 12000 $r \cdot min^{-1}$ for 10 min, 50 µl supernatant was mixed with 450 µl distilled water and 2.5 ml anthrone reagent, and boiled for 10 min. The absorbance value of the solution was measured at wavelength 620 nm. SSC ($\mu g \cdot g^{-1}$) was calculated using the following formula:

$$W_1 \times V_1 \times \text{dilution factor}/(V_2 \times W_2 \times 10^6)$$

Where, W_1: SSC from the standard curve (µg), V_1: Extraction volume (ml), V_2: Test sample volume (ml), W_2: Fresh sample weight (g).

D. Root activity was measured as the reference (19): 0.1 g fresh roots were harvested, cleaned and mixed with 2.5 ml 0.4 % 2,3,5-triphenyltetrazolium chloride in dark for 24 h. The root samples were washed with distilled water and soaked in 5 ml 95 % ethanol at 60 °C water bath for 4 h. Absorbance of the soaked solution was measured at 490 nm. The triphenyl formazan (TTF) content was compared with the standard curve and computed for the root vitality ($mg \cdot g^{-1} \cdot h^{-1}$) by TTF content/root fresh weight.

3. Statistical analysis: Three replicates were used for each experiment. SPSS 20.0 was used for multiple comparison of data to analyse the significant differences of tall fescue characters under treatments labelled with different lowercase letters.

4. Allelopathic effects analysis

Response index (RI) for each trait/variety was calculated as per the method of Williamson and Richardson (17) as under:

$$RI = 1\text{-}T0/T1$$

Where, T0: Control (Without mixture); T1: Mixed sown variety

If IR > 0 : Allelopathic effect positive in mixed sowing, IR < 0 : Allelopathic effect negative in mixed sowing.

RESULTS AND DISCUSSION

1. Allelopathic compounds in root aqueous extracts of *Millennium2* and *Arid3*

GC-MS analysis show that (Table 1) the *Festuca arundinacea* Schreb. *Millennium2* and *Arid3* root aqueous extracts contain15 and 22 chemicals. The 1,1-diethoxy-3-methylbutane contents was maximum i.e. 49.55 % *in Millennium2* and 41.70 % in *Arid3*. It is similar to previous report in popular root extracts that had the high content 23.68 % of 1,1-diethoxy-3-methylbutane (10), indicating it might be significant for the plant root regulation. Further, both grass varieties had more alkane compounds (3), which play important roles in biological transformation such as in Wetland plants (4), *Nicotiana tabacum* L. (21), *Polygonatum odoratum* (Mill.) Druce (18) and *Capsicum frutescens* L. (6).

Table 1. The allelopathic compounds contents in root aqueous extract of *Millennium2* and *Arid3* by GC-MS

Species	Retention Time (min)	Chemical Compound	Area (%)
Arid3	3.392	1,1-Diethoxybutane	2.20
	3.533	2-Methylpentanoyl 2-methylpentanoate	2.20
	3.875	1,1-Diethoxy-3-methylbutane	41.70
	4.133	Octamethylcyclotetrasiloxane	2.72
	4.625	2,6-Dimethylnonane	0.83
	4.967	3-Ethyl-3-methylheptane	3.13
	5.025	3,7-Dimethyldecane	1.38
	5.492	6-Ethyl-2-methyloctane	1.34
	6.45	Dodecane	1.48
	7.05	5-Methylundecane	2.82
	7.183	4,6-Dimethyldodecane	4.47
	7.625	Heptadecane	3.95
	7.792	3-Methyl-5-propylnonane	1.41
	8.008	10-Methylicosane	0.86
	8.342	2,6,10,15-Tetramethylheptadecane	1.93
	8.525	4-Methyltetradecane	1.51
	9.15	1-Iodohexadecane	7.12
	9.242	5-Ethyl-5-methylnonadecane	2.54
	9.342	3,5-Di-tert-butylphenol	6.22
	9.608	Henicosane	5.40
	11.817	Icosane	3.03
	12.617	Tetrapentacontane	1.79
Millennium2	3.392	1,1-Diethoxybutane	2.60
	3.533	2-Methylpentanoyl 2-methylpentanoate	2.22
	3.875	1,1-Diethoxy-3-methylbutane	49.55
	4.133	Octamethylcyclotetrasiloxane	3.57
	4.967	6-Ethyl-2-methyloctane	3.16
	5.025	5-Methylundecane	1.09
	5.425	4-Ethylundecane	2.71
	5.708	Decamethylcyclopentasiloxane	2.37
	7.05	2,3,7-Trimethyldecane	1.62
	7.183	4,6-Dimethyldodecane	4.03
	7.617	3,8-Dimethylundecane	3.39
	9.15	5-Isobutylnonane	10.57
	9.25	9-Methylheptadecane	3.20
	9.6	Henicosane	6.09
	11.825	Icosane	3.84

Two varieties of tall fescue shared 9 chemicals: 1,1-Diethoxybutane, 2-methylpentanoyl 2-methylpentanoate, 1,1-diethoxy-3-methylbutane, 6-ethyl-2-methyl octane, 5-methylundecane, 4,6-dimethyldodecane, henicosane, icosane and octamethylcyclotetrasiloxan. Their contents were similar in both varieties with several exceptions. The 5-methylundecane content in *Arid3* was 2.59 folds higher than in *Millennium2*，6-ethyl-2-methyloctane in *Millennium2* was 2.36 folds higher than *Arid3*. The *Arid3* and *Millennium2* cultivar individually contained 13 and 6 unique chemicals.

Each allelopathic compound might play specific roles in plant growth and development. The ferulic acid, chlorogenic acid, and protocatechuic acid increased the chlorophyll and carotenoid contents of *Rhododendron delavayi* Franch seedlings, but inhibited the accumulation of biomass. Ferulic acid proved more toxic than chlorogenic acid and protocatechuic acid for *Rhododendron delavayi* seedlings (5). Therefore, the differences in chemical composition of root extracts between two grass varieties may contribute to each other growth, which is important for turfgrass composition, prolonging the turfgrass green period and increasing the landscape effects. Thus, selection of varieties and understanding their allelochemicals composition should be seriously considered in mixed sowing in lawns.

II. Mixe sowing effects on tall fescue plants growth

Along with the mixed sowing proportion of two varieties, the physiological indices of grasses varied significantly (Table 2). Mostly, the MDA content and REL of *Millennium2* decreased at low proportion of *Arid3* and gradually improved with the increased proportion, while, the soluble sugar content and root activity increased at low proportion and decreased at high proportion. All the four indices of *Millennium2* performed best at the mixed sowing proportion of 4:1 *(Millennium2*: *Arid3*). But, when the mixed sowing proportion was over this proportion, inhibitory effects occurred. Even, the soluble sugar content and the root activity was 15 % and 12 % lower at proportion of 1:8 than proportion of 4:1. Similarly, with the increase in proportion of mixed sowing *Millennium2*, MDA content and REL of *Arid3* showed the decreasing trend and then gradually increased (such as MDA content was 4.82, 4.52, and 4.84 $\mu mol \cdot g^{-1}$ at the mixed sowing proportion of 1:0, 4:1, and 1:8 of *Arid3*:*Millennium2*), while soluble sugar content (SSC) and root activity showed stimulation and then inhibition (such as SSC was 0.39, 0.43, and 0.39 $\mu g \cdot g^{-1}$ at the mixed sowing proportion of 1:0, 4:1, and 1:8 of *Arid3*:*Millennium2*). The mixed sowing proportion of 4:1(*Arid3*:*Millennium2*) proved most favourable for the growth of *Arid3*. Obviously, mixed sowing of tall fescue varieties could really change the physiological state of both varieties to a certain extent just depending on their proportion.

Several other studies also support this opinion. For example, He (7) reported that the amount of underground biomass of the mixed sowing grasses was increased from 0.042 $g \cdot cm^{-2}$ to 0.073 $g \cdot cm^{-2}$ in the 8:2 and 2:8 proportion of bluegrass and tall fescue at the same seed rate. While, the aboveground biomass was increased and reached the highest in the 2:8 proportion of bluegrass and tall fescue and then decreased. When the proportion of tall fescue in the mixed sowing was > 60 %, the lawn density, texture and aboveground plant biomass remained stable. Thus, the proportion of mixed sowing varieties is important for practical use.

Previous studies had shown the concentration dependent effects of allelopathy. For example, *Trifolium repense* aqueous extracts seriously affected the seeds germination and seedling growth of three turf grasses (*Poa pratensis* L., *Festuca arundinaces* Schreb., *Lolium perenne* L.) by promotion at low concentration and inhibition at high concentration (12). *Millennium2* leaf extracts showed similar concentration effect on different tall fescue varieties (20). Accordingly, when the mixed sowing varieties were less, a little quantity of allelopathic chemicals released could efficiently improve the growth of main varieties. When the mixed sowing varieties were more, the high content of extracts would probably inhibit growth of main varieties. Therefore, the effects of mixed sowing proportion are consistent with the contribution of the allelopathic chemicals from each mixed sowing variety, which might change the chemicals concentration.

Table 2 Physiological indices of *Millennium2* and *Arid3* in various mixed sowing proportions

Variety	Mix sowing ratio of *Millennium 2/Arid3*	MDA （μmol·g-1 FW)	SSC （g·100g-1 FW)	Root activity （mg·g-1 FW·h-1）	REL （%）
Millennium2	1:0	4.70±0.43b	0.38±0.06c	5.60±0.40ab	0.18±0.02a
	8:1	4.36±0.34d	0.41±0.09b	5.93±0.36a	0.16±0.04a
	4:1	4.21±0.06d	0.41±0.01b	5.99±0.44a	0.16±0.04a
	2:1	4.31±0.15d	0.40±0.02b	5.94±0.44a	0.16±0.02a
	1:1	4.58±0.45c	0.38±0.05c	5.79±0.75a	0.16±0.05a
	1:2	4.63±0.57b	0.38±0.01c	5.78±0.31ab	0.17±0.04a
	1:4	4.67±0.21b	0.37±0.08d	5.64±0.48ab	0.17±0.02a
	1:8	4.81±0.62a	0.35±0.03d	5.28±0.72b	0.18±0.05a
Arid3	8:1	4.84±0.45a	0.39±0.01c	5.47±0.24a	0.18±0.03a
	4:1	4.60±0.42b	0.40±0.02b	5.70±0.28a	0.18±0.03a
	2:1	4.64±0.52b	0.41±0.01b	5.70±0.25a	0.17±0.06a
	1:1	4.61±0.5b	0.42±0.01a	5.78±0.20a	0.17±0.03a
	1:2	4.60±0.38b	0.42±0.01a	5.90±0.71a	0.17±0.03a
	1:4	4.52±0.35c	0.43±0.06a	5.90±0.51a	0.17±0.02a
	1:8	4.57±0.53c	0.40±0.03b	5.81±0.38a	0.17±0.02a
	0:1	4.82±0.33a	0.39±0.05c	5.50±0.61a	0.18±0.03a

Note: Values labelled with different lowercase letters in the same row indicate significant differences among the treatments.

III Allelopathic effect of the mixed sowing of grasses

Response index (RI) well reflects the allelopathic effects. Our data showed that the RI value of MDA content, REL, SSC and root activity of mixed sowing *Millennium2* was -0.10, -0.11, 0.08, and 0.07 in mixed sowing proportion of 4:1 (*Millennium2*:*Arid3*), respectively, while it was 0.02 and 0.00, -0.08 and -0.06 in proportion of 1:8. Relatively, the RI value of MDA content, REL, SSC and root activity of *Arid3* was -0.06, -0.06, 0.10, and 0.07 in mixed sowing proportion of 4:1 (*Arid3*:*Millennium2*), and 0.00, 0.00, 0.00, and -0.01 in proportion of 1:8. Obviously, the response of both varieties to mixed sowing was different, *Millennium2* proved more sensitive than *Arid3* (Fig. 3).

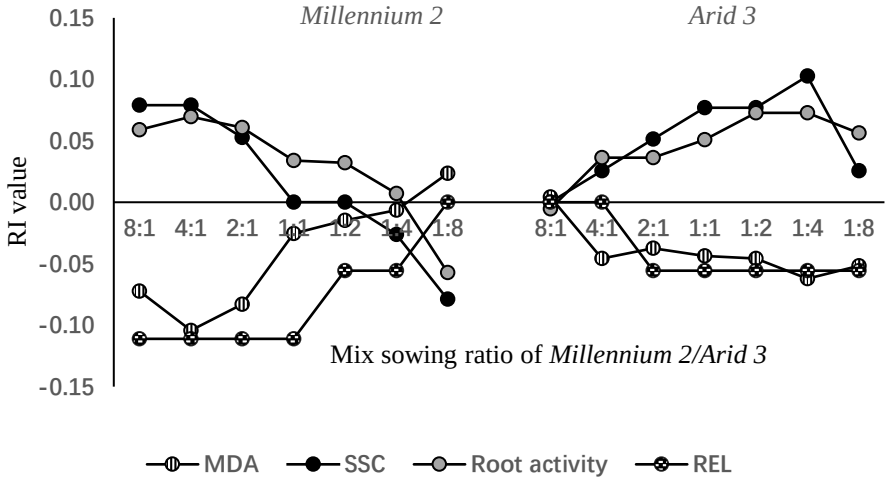

Figure 3. Response index of *Millennium2* and *Arid3* in various mixed sowing proportions

The previous studies provided more evidences for this conclusion, that different varieties have variable allelopathic responses. For example, Green gram could significantly improve the length and yield of Cotton roots, but Sesame inhibits that of Cotton. The leguminous plants are used as companion plants to improve the crop yield of non-legume component (13). Three grasses (Kentucky bluegrass, Tall fescue and Perennial ryegrass) were treated with the root extracts of *Festuca arundinacea*. The root extracts had strong allelopathic inhibitory effects on Kentucky bluegrass followed by Tall fescue and Perennial ryegrass (i.e. % inhibition of 56.85, 15.48 and 13.28 % at 100 mg/mL concentration, respectively) (25). Wang (16) reported the allelopathic effects of 8- *Poa pratenis* varieties on perennial ryegrass seedlings, the variety 'Kentucky' was allelopathically most inhibitory to germination, seedling root development, seedling length and fresh weight followed by 'Platini' and 'Park′ variety. The root exudates of '*Baoding*' *Bermudagrass* stimulated the seed germination, biomass and physiological indices of *F. arundinacea,* there was stimulation at lower concentrations but inhibition at higher concentrations. The combined effects of the absolute RI values on 4-physiological indices (relative electrolyte leakage, MDA content, soluble sugar content, root activity) of *F. arundinacea* cv 'Justice' at 0.75 $mg \cdot ml^{-1}$ concentration RI value was 0.19, while, it was 0.97 in variety 'Greenlabel' (11). Meanwhile, the combined effects on root activity of 6-*Poa pratensis* varieties RI value was 0.50, while it was 0.60-1.15 in leaf indices. It indicated that the response of *F. arundinacea* to '*Baoding*' *Bermudagrass* root exudates was specific to cultivars and tissues. Appropriate species/varieties should be identified for the success of mixed sowing of crops.

CONCLUSIONS

The main variety *Millennium2*, showed improved physiological indices and better growth in mixture ratio of 4:1. The tall fescue varieties *Millennium2* and *Arid3* differed in chemicals composition of root exudates. The variety *Millennium2* was more sensitive to mixture than *Arid3*. GC-MS analysis showed that the root aqueous extracts of *Millennium2* and *Arid3* contained 15 and 22 allelopathic compounds, respectively, and the content of 1,1-diethoxy-3-methylbutane was highest. The selection of compatible species/varieties and their suitable proportion is very important for ideal mixture to improve the appearance of lawn and ecological effects.

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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