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Reduction of Sudden Death Syndrome Foliar Symptoms and *Fusarium virguliforme* DNA in Roots Inoculated With *Rhizophagus intraradices*

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Abstract

There is increasing interest in incorporating arbuscular mycorrhizal fungi (AMF) into agricultural production because of the benefits they provide, including protection against pathogens and pests. Sudden death syndrome (SDS) of soybean is a devastating disease caused by the soilborne pathogen *Fusarium virguliforme.* Multiple management methods are needed to control SDS. The relationship between *F. virguliforme* and AMF is not well documented. The goal of this study was to determine whether soybean plants co-inoculated with *F. virguliforme* and the AMF species *Rhizophagus intraradices* showed reduced SDS foliar symptom severity and reduced relative *F. virguliforme* DNA quantities in soybean roots. Six soybean genotypes were inoculated with *F. virguliforme* alone or with *R. intraradices* in a greenhouse experiment. Averaged over the six soybean genotypes, area under the disease progress curve values and relative *F. virguliforme* DNA quantities were 45 and 28% lower ($P < 0.05$), respectively, in roots co-inoculated with *R. intraradices* compared with roots of control plants inoculated with *F. virguliforme* only. Weight of roots co-inoculated with *R. intraradices* were 58% higher ($P < 0.05$) compared with roots of plants not inoculated with *R. intraradices*. Nutrient analysis showed higher boron, phosphorus, potassium, sodium, and sulfur concentrations in root tissues of plants co-inoculated with *R. intraradices* compared with plants inoculated with *F. virguliforme* ($P < 0.05$). Overall, this study showed that *R. intraradices* reduced SDS severity and relative *F. virguliforme* DNA quantities while simultaneously increasing growth and nutrient uptake of plants. Further testing of AMF inoculants in the field will indicate whether incorporating them into soybean SDS management practices will reduce the impact of SDS on soybean production.

Keywords: arbuscular mycorrhizal fungi, soybean, sudden death syndrome

Erratic weather patterns and warming temperatures caused by climate change may provide more conducive environments for pathogens and pests to attack plants and reduce yields (Chakraborty and Newton 2011). However, concern about the environmental impacts of current agricultural practices has led to increased interest in alternative methods to manage plant diseases and pests and to support growth and yield while reducing the need for pesticides. One alternative method of disease management uses beneficial microbes, such as arbuscular mycorrhizal fungi (AMF) (Azcón-Aguilar and Barea 1997). AMF are obligate biotrophs found in the soil that form a symbiotic relationship with 80% of land plants, and they are known to facilitate nutrient uptake, increase drought tolerance, and increase protection against pathogens and pests (Berruti et al. 2016).

AMF are known to have positive effects on plant protection against soilborne necrotrophic pathogens (Pozo et al. 2010; Whipps 2004) and certain plant parasitic nematodes (Hol and Cook 2005). Even when AMF do not reduce disease severity, mycorrhiza-infected mycorrhizal plants can show greater biomass and yield compared with nonmycorrhizal controls (Gernns et al. 2001; Meyer and Dehne 1986). To some extent, the degree of bioprotection provided by AMF may be host genotype dependent. For example, a study of common bean and tomato genotypes showed that only some genotypes benefited from the protection of AMF colonization against white mold (*Sclerotinia sclerotiorum*) and bacterial spot (*Xanthamonas campestris* pv. *vesicatoria*), respectively (Mora-Romero et al. 2015). Variation in the degree of Glanville fritillary caterpillar feeding was observed on different lines of ribwort plantain colonized with a mixture of native AMF (Rasmussen et al. 2017). Mycorrhizal enhancement of root defense chemicals was also observed to be genotype dependent in ribwort plantain (De Deyn et al. 2009).

Genetic factors contributing to increased growth benefits have been identified in *Allium* spp. (Galván et al. 2011) and maize (Kaeppler et al. 2000). Genetic variation within a host has been shown to affect bioprotection of mycorrhizae against pathogens in strawberries against *Phytophthora fragariae* and in tomatoes against *Fusarium oxysporum* f. sp. *lycopersici* (Mark and Cassells 1996; Steinkellner et al. 2012). Breeding for increased mycorrhizal symbiosis could be instrumental to increase plant protection against diseases and pests (Hohmann and Messmer 2017).

Sudden death syndrome (SDS) of soybean is a devastating disease caused by the soilborne fungus *Fusarium virguliforme*. SDS is considered one of the most important soybean diseases in the United States, with a potential to cause 100% yield loss in conducive conditions (Hartman et al. 2015a). This disease is also found in Brazil, Malaysia, and South Africa (Hartman et al. 2015a). There currently are very few management methods for controlling this disease. Although host resistance is considered the most effective method, complete resistance has not been identified (Hartman et al. 2015a, b).

Little information is known about the relationship between mycorrhizal fungi and *F. virguliforme*. A greenhouse study reported that when AMF species *Funneliformis mosseae* was added to soils

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infested with *F. solani*, plant height and root weight were greater than those of the noninoculated plants (Zambolim and Schenck 1983). An in vitro study found that AMF species *Rhizophagus irregularis* decreased soybean root colonization of *F. virguliforme* (Giachero et al. 2017). However, both studies used only one soybean genotype, and reports indicate that colonization of AMF species *R. intraradices* in soybean is genotype specific (Khalil et al. 1994; Pawlowski et al. 2019). Evaluating multiple soybean genotypes is needed to better understand AMF-mediated protection against *F. virguliforme* foliar symptoms and root colonization in soybean.

The overall goal of this study was to determine whether different soybean genotypes vary in response to *F. virguliforme* when co-inoculated with *R. intraradices*. The specific objectives were to determine whether inoculating soybean plants with *R. intraradices* reduced SDS foliar symptom severity and reduced relative *F. virguliforme* DNA quantities in soybean roots, affected root weight and nutrient composition in roots colonized by *F. virguliforme*, and produced responses that were dependent on soybean genotypes.

Materials and Methods

Soybean genotypes. Six different soybean cultivars were selected based on differences in root colonization or AMF-mediated aboveground growth response shown in previous experiments, and all were identified as susceptible to *F. virguliforme* (Pawlowski et al. 2019). Soybean plant introductions (PIs) were provided by the U.S. Department of Agriculture Agricultural Research Service Soybean Germplasm Collection (Urbana, IL). Soybean breeding lines (LD) were provided by Dr. Brian Diers (University of Illinois) (Table 1).

Mycorrhizal inoculum. To increase *R. intraradices*, a modified sheared root method was used (Sylvia and Jarstfer 1992). Cone-shaped containers were plugged with a cotton ball and filled with 100 ml of sterilized torpedo sand. A 2.5-cm (15-ml) layer of inoculum, original stock from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (invam.wvu.edu), was spread evenly in the cone and covered with another 2.5 cm of torpedo sand. Seeds of soybean cultivar Williams 82 were sown 2 cm deep into the sand, one seed per cone. Plants were watered regularly and grown for 6 weeks in a greenhouse held at a constant 25°C with a 16-h photoperiod. Six weeks after planting, containers were air dried for 5 days, and roots were collected for inoculum. Roots infested with AMF were cut into 1-cm fragments with scissors and stored for 1 week in a cold room set at 4°C.

***F. virguliforme* inoculation.** Inoculum was prepared by previously established methods (Chawla et al. 2013) with *F. virguliforme* isolate Mont-1, a originating from Monticello, Illinois. The isolate was grown on potato dextrose agar in the dark at 24°C for 14 days before use. A liter of sorghum seed was soaked in 1.5 liters of water overnight, then drained, placed in gusseted bulk spawn bags, and autoclaved 60 min on two successive days. Upon cooling, sorghum seed was infested with one petri dish of *F. virguliforme* by cutting the infested agar into 1-cm^2 sections with a sterile scalpel and transferring into the bag of autoclaved sorghum seed. Bags were heat sealed and incubated at room temperature for 2 weeks, with gentle mixing every few days. Inoculum was then air dried at room temperature for 2 days and stored in paper bags in a cold room at 4°C.

Experimental design. The experiment was designed as a factorial arranged in a completely randomized design with five replicates. Factors included soybean accession (six accessions) and mycorrhizal inoculation (with and without) for a total of 60 experimental units. Five seeds of each genotype were sown into 15-cm azalea pots filled with torpedo sand. All experimental units were inoculated with *F. virguliforme* by spreading (80 ml) of a sand/infested sorghum grain mixture (3:1 ratio) evenly 2.5 cm below the surface of the sand. Half of the pots were co-inoculated with *R. intraradices* by incorporating 50 spores per pot into the *F. virguliforme* sorghum inoculum layer. The experiment was repeated with different randomization of each treatment. For both trials, roots from two replications of each genotype were stained to verify mycorrhizal colonization. Root tissues were cleared with a 20% bleach solution at room temperature for 16 h, rinsed three times with water, and soaked in a 2% HCl solution for 30 min before being soaked in 0.05% chlorazol black E in 1:1:1 lactic acid, glycerol, and water solution for 48 h (Pawlowski et al. 2019). Stained tissues were then placed in a Petri dish under a light microscope to observed fungal structures including arbuscules and spores.

Foliar disease severity rating scale. Plants were evaluated for foliar symptoms 14, 17, and 21 days after planting with a 1 to 8 foliar disease severity rating scale (Chawla et al. 2013). For example, plants were considered a 1 if they showed no symptoms, a 4 if >2 cm of the leaf margins were necrotic, and an 8 if the plant was entirely defoliated.

Root weight measurements and nutrient analysis. At 21 days after inoculation, roots from each pot were excised, washed, and lyophilized for 72 h. Dry weights were recorded, and root masses were ground with a basic microfine grinder for *F. virguliforme* DNA extraction and nutrient analysis. Because there were no interactions between genotype and mycorrhizal treatment, root samples of two soybean accessions, PI 071465 and LD13-6678, were chosen at random and submitted for nutrient analysis via inductively coupled plasma–mass spectrometry.

Quantification of *F. virguliforme*. DNA extractions were completed using a DNA isolation kit on 25 mg of dried ground root tissue per experimental unit. Quantitative PCR assays were performed with MxPro version 4.1 software. Assays were conducted with primers Fv-Li-F (5′-GGCTGAACTGGCAACTTGGA-3′) and Fv-Li-R (5′-CAAAGCTTCATTCAATCCTAATACAATC-3′), with two technical replicates of each experimental unit (Li et al. 2008). The soybean reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified with primers GmGAPDH v14.fwd (5′-CATCGGAGGGAAGTATGAAAGG-3′) and GmGAPDH v14.rev (5′-GTACAATGCATGATGGTGGC-3′) (Haudenshield and Hartman 2011). Relative quantities of *F. virguliforme* DNA in each treatment

Table 1. Soybean genotypes used to evaluate the interaction between arbuscular mycorrhizal fungal species *Rhizophagus intraradices* and *Fusarium virguliforme,* the causal pathogen of sudden death syndrome (SDS)

Genotype[v]	Acquisition or release date[w]	Mycorrhizal colonization[x]	Mycorrhizal responsiveness (%)[y]	SDS[z]
LD10-10198	2007	n/a	108	Susceptible
LD13-6678	2012	n/a	60	n/a
PI 71465	1927	Mid	n/a	Susceptible
PI 91159-4	1931	High	n/a	Susceptible
PI 602502B	1997	Low	8	Susceptible
PI 605765B	1998	Low	25	Susceptible

[v] All plant introduction (PI) accessions were obtained from the U.S. Department of Agriculture Soybean Germplasm Collection housed at the University of Illinois in Urbana. Breeding lines (LD) were obtained from Dr. Brian Diers at the University of Illinois in Urbana.

[w] Acquisition dates for PIs at https://www.ars-grin.gov/.

[x] Colonization by arbuscular mycorrhizal fungus species *R. intraradices* (Pawlowski et al. 2019).

[y] Genotypes were tested for their difference in aboveground plant growth between *R. intraradices* inoculated and noninoculated plants (Pawlowski et al. 2019).

[z] Before the current study, LD10-10198 was determined to be susceptible in uniform field trials in 2015 and greenhouse evaluations, and PIs were determined susceptible in greenhouse evaluations via the modified inoculum layer technique (Pawlowski et al. 2019).

were measured as the amount of *F. virguliform*e DNA per nanogram of GAPDH DNA.

Statistical analysis. Area under the disease progress curve (AUDPC) values were calculated before analyses with average ratings per pot for 14, 17, and 21 days after inoculation. Root mass dry weight was divided by the number of plants per pot to obtain average weight per plant. Relative *F. virguliforme* concentration data were transformed by taking the log to base 10 to correct for a Poisson distribution of the residuals. Bartlett's test for homogeneity of variance was performed before trials were combined. An analysis of variance was run on AUDPC, root weights, relative *F. virguliforme* quantities, and nutrient concentrations. Means were separated via the least square means Student's *t* procedure at $\alpha = 0.05$. All statistical analyses were completed in JMP version 14.0.

Fig. 1. Foliar symptoms (top) and roots (bottom) of soybean plants 21 days after inoculation with *Fusarium virguliforme* with (left) or without (right) inoculation of arbuscular mycorrhizal fungus *Rhizophagus intraradices*.

Table 2. Analysis of variance summary showing *F* ratios for area under the disease progress curve (AUDPC), root dry weights, and quantities of *Fusarium virguliforme* of six soybean genotypes grown in a greenhouse inoculated with *F. virguliforme* with or without mycorrhizal species *Rhizophagus intraradices*[y]

Source of variation[z]	*df*	AUDPC	Root weight	Relative *F. virguliforme*
Trial	1	0.07	2.32	0.57
Soybean genotype	5	9.46***	2.41*	3.86**
Trial × soybean genotype	5	3.05*	0.30	9.09***
Mycorrhizal treatment	1	177.79***	38.11***	6.65*
Trial × mycorrhizal treatment	1	17.40***	0.96	2.52
Soybean genotype × mycorrhizal treatment	5	1.26	0.81	0.10
Trial × soybean genotype × mycorrhizal treatment	5	1.87	0.69	0.37

[y] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant; *df*, degrees of freedom.
[z] Trial, soybean genotype, and mycorrhizal treatment were all fixed effects.

Results

General analysis. There was no significant ($P > 0.05$) difference in variance for any of the measured variables, and data for the trials were combined. There were visible differences in foliar severity and root mass between the mycorrhizal and nonmycorrhizal controls for all genotypes (Fig. 1). For both trials, fungal structures, including arbuscules and spores, were observed in stained roots inoculated with *R. intraradices* in each of two replications but not in plants not inoculated with *R. intraradices*.

Foliar severity ratings. For AUDPC values, there was a significant ($P < 0.01$) interaction between trial and mycorrhizal treatment effects (Table 2). However, for both trials, AUDPC values were lower ($\alpha = 0.05$) in plants inoculated with *R. intraradices* compared with nonmycorrhizal control plants with a 57 and 33% reduction in AUDPC values for trials 1 and 2, respectively (Table 3).

Root weights. There was no interaction between soybean genotype and mycorrhizal treatment, but there was a mycorrhizal treatment effect ($P < 0.001$) on root weights (Table 2). Average root weight of plants inoculated with *R. intraradices* (153 mg per pot) was 58% greater than that of noninoculated *R. intraradices* plants (97 mg per pot) (Table 3).

***F. virguliforme* quantification.** There was no interaction between soybean genotype and mycorrhizal treatment, but there was a significant ($P < 0.001$) mycorrhizal treatment effect (Table 2). Plants inoculated with AMF had an average of 27.5 pg of *F. virguliforme* DNA/ng GAPDH DNA, compared with an average of 38.2 pg of *F. virguliforme* DNA/1 ng GAPDH DNA (Table 3) for noninoculated *R. intraradices* plants.

Nutrient analysis. There was a significant mycorrhizal treatment main effect for phosphorus, potassium, sulfur, boron, and sodium concentrations (Table 4). Mycorrhizal treatment significantly ($P < 0.05$) increased the concentrations of all five nutrients by 15, 78, 33, 39, and 50%, respectively (Table 5).

Discussion

This the first study to evaluate a panel of soybean genotypes for AMF-mediated disease protection against SDS. Although there were significant differences between genotypes for AUDPC, root weight, and relative quantities of the soybean fungal pathogen *F. virguliforme*, there was no significant interaction between genotype and mycorrhizal treatment, indicating a similar level of benefit across genotypes. Previous studies in other pathosystems have reported significant genotype by pathogen by AMF interactions and, in general, showed that cultivars more susceptible to the pathogen gained more benefit from AMF infection than less susceptible cultivars (Mark and Cassells 1996; Ronsheim 2016; Steinkellner et al. 2012). In a preliminary experiment, we did observe a significant genotype by mycorrhizal treatment interaction in AUDPC values. Two moderately resistant soybean cultivars showed no significant difference between the mycorrhizal and nonmycorrhizal controls. All susceptible cultivars in the same test showed significantly lower AUDPC values between mycorrhizal treatments (Supplementary Fig. S1). Because the current study selected only cultivars known to be susceptible to *F. virguliforme*, we did not see a genotype-dependent response indicating AMF-mediated protection against the pathogen. The results from the panel of susceptible cultivars and previous studies indicate that genotype-dependent protection may be influenced more by the host's susceptibility to the pathogen than by the relationship with the beneficial fungi.

Several proposed mechanisms may be at play during AMF-mediated protection against root pathogens. One proposed mechanism is that AMF associations result in overall increase in plant

Table 3. Average values of area under the disease progress curve (AUDPC), root dry weights, and quantities of *Fusarium virguliforme* of six soybean genotypes grown in a greenhouse inoculated with *F. virguliforme* with or without mycorrhizal species *Rhizophagus intraradices*

Treatment	AUDPC trial 1[w]	AUDPC trial 2	Root weight (mg)[x]	Relative *F. virguliforme*[y]
Mycorrhizal	14.8 a[z]	19.3 a	152.8 a	27.5 a
Nonmycorrhizal	35.0 b	28.9 b	97.2 b	38.2 b

[w] AUDPC values for disease severity of sudden death syndrome were calculated with foliar symptom ratings taken 14, 17, and 21 days after planting.
[x] Dry root weights per plant of soybean plants with mycorrhizal or nonmycorrhizal treatments. Root masses of five soybean plants per pot were excised, washed, and lyophilized for 72 h before measurements.
[y] Relative (picograms of *F. virguliforme* DNA per nanogram of soybean DNA) quantities of *F. virguliforme* in root tissues. Root masses of five soybean plants per pot were excised, washed, lyophilized for 72 h, and ground up before quantification. Both *F. virguliforme* and soybean DNA quantification assays used were previously developed (Haudenshield and Hartman 2011; Li et al. 2008).
[z] Numbers within a column followed by a different letter differ at $\alpha = 0.05$.

Table 4. Analysis of variance summary showing *F* ratios for nutrient concentrations of root samples of two soybean genotypes grown in a greenhouse inoculated with *Fusarium virguliforme* with or without mycorrhizal species *Rhizophagus intraradices*[y]

Source of variation[z]	*df*	Phosphorus	Potassium	Sulfur	Boron	Sodium
Trial	1	8.13***	34.49***	14.00***	1.23	9.21***
Soybean genotype	1	0.24	2.18	0.53	5.94*	1.55
Trial × soybean genotype	1	1.39	0.51	0.01	1.49	0.1
Mycorrhizal treatment	1	5.99*	5.78*	7.28*	12.38***	8.71***
Trial × mycorrhizal treatment	1	3.95	0.43	0.1	3.39	0.02
Soybean genotype × mycorrhizal treatment	1	0.19	0.56	0.19	0.26	0.03
Trial × soybean genotype × mycorrhizal treatment	1	0.001	1.8	0.01	1.22	0.22

[y] *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.
[z] Trial, soybean genotype, and mycorrhizal treatment were all fixed effects.

Table 5. Average nutrient concentrations of root samples of two soybean genotypes grown in a greenhouse inoculated with *Fusarium virguliforme* with or without mycorrhizal species *Rhizophagus intraradices*

Treatment	Phosphorus (%)[y]	Potassium (%)	Sulfur (%)	Boron (ppm)	Sodium (ppm)
Mycorrhizal	0.23 a[z]	1.0 a	0.2 a	26.1 a	0.4 a
Nonmycorrhizal	0.20 b	0.6 b	0.1 b	18.6 b	0.3 b

[y] Nutrient concentration analysis was performed on soybean root tissues via inductively coupled plasma–mass spectrometry.
[z] Numbers within a column followed by a different letter differ at $\alpha = 0.05$.

growth, allowing a host to compensate for damage done by the pathogen (Xavier and Boyetchko 2004). Our study found that in the presence of the pathogen, plants inoculated with *R. intraradices* had 58% more root mass than plants not inoculated with *R. intraradices*. In a field experiment, inoculation with mycorrhizal fungi increased dry root weights by 151% compared with a nonmycorrhizal control in the presence of *F. solani* (Zambolim and Schenck 1983). Previous studies show root weight is negatively correlated with SDS disease severity (Gongora-Canul and Leandro 2011). Therefore, the AMF-mediated root growth shown in our study may be a major component of the reduced disease severity in plants inoculated with *R. intraradices.*

Another mechanism to explain the reduction in SDS severity in plants inoculated with *R. intraradices* could be related to the increase in nutrient uptake. Increased plant nutrition after mycorrhizal colonization has been previously suggested as a mechanism for reducing disease severity (Abdel-Fattah et al. 2011). All but one of the nutrients that increased in mycorrhizal roots in our study have roles in reducing disease severity in plants. Phosphorus is an essential nutrient involved in many metabolic processes and is shown to protect against pathogens where vigorous root growth allows the plant to evade disease (Dordas 2008). A field study found that increasing the rate of phosphorus fertilization reduced SDS disease severity (Adee et al. 2016). However, a greenhouse study found that soils amended with phosphorus increased disease severity by as much as 45% (Sanogo and Yang 2001). AMF-mediated increase of phosphorus in roots of tomato and strawberry had no effect on the severity of *F. oxysporum* f. sp. *radices-lycopersici* and *F. oxysporum* f. sp. *fragariae*, respectively (Caron et al. 1986; Matsubara et al. 2004). There is no specific evidence that the increase in phosphorus contributed to a reduction in disease severity in our study, but more research should be done to determine whether there is a relationship between phosphorus and SDS severity. The significant increase in potassium compared with other nutrients is of interest based on previous findings between the interaction of potassium and *F. virguliforme*. A previous study showed a significant reduction in SDS severity when potassium chloride was applied to soils at planting (Sanogo and Yang 2001). Potassium is known to play a role in epidermal cell wall development, and hardening of the epidermis may make it more difficult for pathogens to invade the plant (Dordas 2008). Both phosphorus and potassium are known to increase root growth and may have aided in AMF-mediated disease suppression. Boron is also known to play a role in plant disease resistance (Dordas 2008). This relationship has not been well studied, but it is proposed to result from the function of boron in promoting cell wall stability (Brown et al. 2002).

Although increased nutrient uptake via AMF association may be a mechanism in reducing disease severity, other factors might include reduction of pathogen infection by direct competition or by stimulating defense responses (Xavier and Boyetchko 2004). A meta-analysis found a reduction in the quantity of a fungal pathogen in the presence of AMF in 65 of 125 experiments (Borowicz 2001). Our study confirmed a significant reduction in the relative quantity of *F. virguliforme*, with 28% less DNA detected in the presence of *R. intraradices* compared with plants not inoculated with *R. intraradices*. One previous study found a 12% reduction in root colonization by *F. virguliforme* when soybean plantlets colonized with *R. irregularis* were grown in Petri dishes for 3 days compared with a control (Giachero et al. 2017). A field study found no reduction of *F. solani* propagules in the presence of mycorrhizal fungi in soil samples of a field test, but the study did not account for the amount of *F. solani* in the root system (Zambolim and Schenck 1983).

In summary, our study showed that inoculating soybean plants with *R. intraradices* reduced both SDS severity and *F. virguliforme* DNA in roots while increasing growth and nutrient uptake of plants. As in other studies that have used AMF to protect plants from pathogens, the reduction in disease severity we observed when pathogen-challenged plants were co-inoculated with *R. intraradices* was probably caused by several benefits provided by AMF. This reduction in disease severity was not shown to be soybean genotype specific, and we infer that when challenged with a pathogen, susceptible genotypes will benefit from colonization of *R. intraradices*. *R. intraradices* and possibly other AMF species could become routine inputs for use in a multimanagement approach to control SDS.

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