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Acetolactate Synthase–Inhibitor Resistance in Yellow Nutsedge (*Cyperus esculentus*): II—Physiognomy and Photoperiodic Response

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Yellow nutsedge is one of the most problematic weedy sedges in rice–soybean systems of the Mississippi Delta region. An acetolactate synthase (ALS)-inhibiting, herbicide-resistant (*Res*) yellow nutsedge biotype was recently documented in eastern Arkansas, which showed intermediary growth habit between yellow nutsedge and purple nutsedge and also exhibited differential photoperiodic sensitivity to flowering. The objectives of this study were to: (a) determine variation in reproductive characteristics of the *Res* biotype and three susceptible (*Sus*) yellow nutsedge biotypes, (b) understand the influence of photoperiod on growth and reproduction, (c) understand the potential role of seeds in population establishment, and (d) elucidate the phylogenetic relationships between the *Res* yellow nutsedge biotype and purple nutsedge. Tuber production per plant and tuber weight of the *Res* biotype were less than that of the *Sus* biotypes. Differences in quantitative traits, such as shoot and tuber production existed between the *Res* and *Sus* biotypes for photoperiods ranging from 12 to 16 h. Generally, photoperiods greater than 12 h increased shoot development in all yellow nutsedge biotypes, with differential responses among the biotypes. Number of tubers reached the maximum for the *Res* biotype at a 14-h photoperiod. Over a 90-d period, inflorescence formation was only observed in the *Res* biotype with maximum flowering and seed production in the 14-h photoperiod. Subsequent tests revealed up to 18% seed germination, suggesting that seed could also play a role (in addition to tubers) in the persistence and spread of the *Res* yellow nutsedge. Phylogenetic analysis based on ribosomal DNA internal transcribed spacer (ITS) regions and mitochondrial *nad4* gene intergenic spacer sequences indicated that the *Res* biotype was more closely associated with *Sus* yellow nutsedge biotypes. Nevertheless, 100% similarity for the *nad4* gene sequences between the *Res* yellow nutsedge biotype and a reference purple nutsedge suggests that the *Res* biotype is likely a result of hybridization between yellow and purple nutsedges, which perhaps explains the intermediary growth characteristics observed in the *Res* biotype.

Nomenclature: Purple nutsedge, *Cyperus rotundus* L.; yellow nutsedge, *Cyperus esculentus* L.; rice, *Oryza sativa* L.; soybean, *Glycine max* (L.) Merr.

Key words: ALS-inhibiting herbicide-resistant perennial weed, hybridization, internal transcribed spacer (ITS), mitochondrial *nad4* gene, photoperiodic response, phylogeny, quantitative traits.

Yellow nutsedge is one of the most important perennial herbaceous members of the Cyperaceae family with a C_4 photosynthetic pathway (DeFelice 2002). It is a tuber-forming sedge endemic in Eurasia and reported to be naturalized within the United States by 1850s (Bartlett 1889). Yellow nutsedge is an invasive and a persistent weed in well-irrigated agricultural and horticultural crops (Ransom et al. 2009; Webster 2005), and a noxious weed in Arkansas rice–soybean rotations (Norsworthy et al. 2013). Chemical control, particularly with acetolactate synthase (ALS)-inhibiting herbicides, in comparison to cultural and mechanical control, has been the most effective means for managing this weed species. Recently, a nutsedge biotype identified as yellow nutsedge by a USDA plant taxonomist (Dr. Charles Bryson) exhibited resistance to several ALS-inhibiting herbicides commonly used in Arkansas rice production (Tehranchian et al. 2014b).

Yellow nutsedge propagates asexually by forming underground tubers at the apical end of creeping fibrous rhizomes (Mulligan and Junkins 1976). It invades farmlands with extensive root and rhizome proliferation and massive tuber production (DeFelice 2002). A three-dimensional spatial model developed by Schippers et al. (1993) suggested that tillage and field machinery are the main causes of yellow nutsedge dispersal in agricultural fields. Tubers usually sprout in late spring, developing a basal bulb and then an aerial shoot. Under suitable conditions,

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mature stands of yellow nutsedge form a hollow foliar tube from the two differentiated leaf primordia at the basal bulb apical meristem (Jansen 1971). Simultaneously, the floral leaf primordia elongates to form an axillary inflorescence, which leads to production of simple or compound umbels containing self-incompatible, out-crossing flowers (DeFelice 2002). For the closely related chufa (*Cyperus esculentus* L. var. *sativus* Boeckeler), seed production can range from 0 to 2,420 at a single axillary inflorescence of some weedy stands (Stevens 1932). Stoller and Sweet (1987) believed that seeds have minor importance in the life cycle, persistence, and demography of yellow nutsedge. Yellow nutsedge seedling establishment has been rarely recorded in the field because young seedlings are easily overlooked and are very sensitive to desiccation, particularly in frequently cultivated areas (Holm et al. 1991; Lapham and Drennan 1990). However, Thullen and Keeley (1979) speculated that seeds were the cause of yellow nutsedge infestations in cotton (*Gossypium hirsutum* L.) fields in California.

Estimations of variability in yellow nutsedge populations based on morphological and phenological traits suggested its sexual reproduction in the United States (Costa and Appleby 1976; Holt 1994; Matthiesen and Stoller 1979). Similarly, genetic diversity studies on yellow nutsedge collected from different geographical locations using polymerase chain reaction (PCR) methods (e.g., randomly amplified polymorphic DNA [RAPD] analysis), also suggested seed as a source of infestation (Okoli et al. 1997). Seeds are extremely dormant when produced, but dormancy can be broken by several months of incubation at 25 C (Holm et al. 1991).

Regeneration of perennial invasive weed species is highly dependent on environmental conditions (Went 1957). A combination of plant physiognomy and phenology "reflecting form–function relationships" (Box 1996) with ecophysiological aspects can be very helpful in developing control measures for perennial invasive weed species (Catford et al. 2014). Photoperiod is one of the main factors that influence yellow nutsedge growth, tuber production, and flowering (Jansen 1971). The promoting effect of photoperiod on tuber and shoot formation has been observed in yellow nutsedge. According to Garg et al. (1967), maximum tuber production of yellow nutsedge compared to shoot growth and flowering can be associated with short photoperiod, low soil nitrogen level, lack of gibberellin, and high temperature.

Previous work revealed drastic differences in growth habit between the ALS-resistant yellow nutsedge (*Res*) biotype originating from eastern Arkansas and the susceptible (*Sus*) biotypes (Bagavathiannan et al. 2015). In particular, the growth and phenological characteristics of the *Res* biotype appeared to be intermediate between yellow and purple nutsedge, with chain-like network of rhizomes and basal bulbs as in purple nutsedge, and formation of solitary, terminal tubers and golden yellow-colored inflorescence (and other morphological characteristics) resembling yellow nutsedge. It was unclear whether the *Res* biotype was a hybrid between yellow and purple nutsedge. Moreover, the *Res* biotype flowered uniformly under greenhouse conditions (30/20 C day/night temperatures and 14-h photoperiod), whereas the susceptible standards failed to do so (Bagavathiannan et al. 2015). It appears that the photoperiodic requirements for reproduction in the *Res* biotype is different from the *Sus* biotypes, and the influence of photoperiod on differences in flowering, tuber production, and seed viability between the *Res* and *Sus* yellow nutsedge biotypes is not well understood.

The objectives of the current study were to (a) determine reproductive attributes of the *Res* biotype compared to *Sus* biotypes, (b) determine the influence of photoperiod on reproduction, (c) understand the potential role of seed production in the establishment of the *Res* biotype, and (d) conduct a phylogenetic analysis using ribosomal and mitochondrial DNA to estimate genetic similarities between the *Res* and *Sus* yellow nutsedge biotypes and a reference purple nutsedge biotype.

Materials and Methods

Plant Material and Propagation. The *Res* biotype was collected from a rice field near Hoxie, AR in 2012 and clonally propagated in the greenhouse at the Agricultural Research and Extension Center, Fayetteville, AR. Plants were confirmed resistant to halosulfuron rates as high as 13,568 g ai ha^{-1} (Tehranchain et al. 2014b). Tubers of *Sus* biotypes originated from (1) Azlin Seed Company in Leland, MS (hereafter referred to as *Sus*-1); (2) a rice field near Stuttgart, AR (hereafter referred to as *Sus*-2); and (3) from a soybean field at the Agricultural Research and Extension Center in Fayetteville, AR (hereafter referred to as *Sus*-3). Tubers of all biotypes were initially sprouted in individual plastic trays (55.5 cm^3 by 26.5 cm^3 by 5.5 cm^3) containing potting mix (LC1, Sun Gro Horticulture, Vilna, Alberta, Canada) in the greenhouse.

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Growth and Tuber Production. A single sprouted tuber of each biotype was transplanted into the center of plastic pots (32 cm long by 19 cm wide by 11 cm deep) containing potting mix in the greenhouse at 30/20 C day/night temperature under 14-h daily photoperiod. Plants were irrigated daily and fertilized weekly with water-soluble fertilizer (Scotts Miracle-Gro, Marysville, OH). The experiment was conducted in a completely randomized design with four replications and two runs from early spring to late autumn 2013. The aboveground biomass was harvested 150 d after transplanting (DAT). Roots and rhizomes (belowground biomass) were washed from the potting mix and separated from the aboveground biomass. Tubers were harvested and counted, and subsequently all biomass was oven dried separately at 60 C for 72-h and dry weight recorded (e.g., root, shoot, tuber). Data were subjected to ANOVA using PROC MIXED in SAS 9.3 version (SAS Institute, Cary, NC). There was no significant difference between the two runs; therefore, data were pooled and means separated using Fisher's protected LSD at $\alpha = 0.05$.

Photoperiod Experiment. This experiment was conducted in growth chambers (CMP 6050; Conviron, Pembina, ND), with four biotypes (*Sus*-1, *Sus*-2, *Sus*-3, and *Res*) and three photoperiods (12-, 14-, and 16-h) under a combination of florescent-incandescent lights that provided approximately 900 μmol m^{-2} s^{-1} of photosynthetically active radiation for the aforementioned photoperiods. Day and night temperatures were 25 and 21 C, respectively as suggested by Jansen (1971). The treatments were arranged in a split-plot design with photoperiods as the main plot and biotypes as the subplot with five replications. The experiment was also repeated over time (i.e., two separate runs). Young plants at the three- to four-leaf stage were transplanted into plastic pots (11 cm tall by 14 cm diam) and moved to growth chambers (one each for the specific photoperiod treatment). Plants were watered and fertilized as required. After 12 wk, matured inflorescences were harvested and number of umbels and seeds per plant were counted. Aboveground biomass was measured by weighing the shoots after oven-drying at 60 C for 72 h. Tubers were also harvested and counted.

Seedling Emergence. Because none of the susceptible biotypes flowered and because of limited seed production by the *Res* biotype, seed viability and germination tests were not conducted. Forty seeds of each replicate per treatment, or the entire seed lot when less than 40 seeds were produced, were sown in plastic pots (13 cm^3 by 13 cm^3 by 6 cm^3) filled with nonsterile potting mix. The experiment was conducted in the greenhouse under the conditions noted earlier. Seeds were sown at a 0.5-cm depth, and seedling emergence was monitored on a regular basis for up to 72 d after sowing as suggested by Lapham and Drennan (1990).

Data Analysis. The whole-plant biomass and tuber production data from the photoperiod experiments were subjected to ANOVA using PROC MIXED in SAS. Data were pooled across the two experimental runs because there were no significant differences. Although tuber production was observed in all the biotypes, flowering occurred only in the *Res* biotype across all the different photoperiods tested in this study. Thus, flowering, seed production, and seedling emergence data were subjected to ANOVA using PROC GLM in SAS for each of the dependent variables mentioned above for each photoperiod treatment. For all tests, normality was tested prior to ANOVA using the PROC UNIVARIATE statement of SAS and test results indicated that data transformations were not required. Following ANOVA, mean separations were performed using Fisher's protected LSD ($\alpha = 0.05$).

Phylogenetic Analysis. *Internal Transcribed Spacer (ITS) Amplification.* Total pure cellular DNA was extracted from 100 mg fresh shoot of each yellow nutsedge biotype and a reference purple nutsedge that was collected from the weed nursery of the University of Arkansas, Fayetteville, AR, using a Qiagen DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands; http://www.qiagen.com). Entire ITS region of the plant DNA samples were amplified using ITS1 (5′-TCCGTAGGTGAACC TGCGG-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) primers designed by White et al. (1990). The PCR reactions were prepared in 25 μL containing ~ 20 ng of DNA, 12.5 μL GoTaq Green master mix (Promega US, Madison, WI), 0.5 μL of each primer (18 μM), and 9.5 μL of nuclease-free water. A Bio-Rad Thermal Cycler T100 (Bio-Rad, Hercules, CA) was programed based on Tehranchian et al. (2014a) for 10 min initial denaturation at 94 C followed by 35 cycles of denaturation at 94 C for 30 s, annealing at 51.7 C for 1 min and 1 min extension at 72 C, with a final extension of 10 min at 72 C.

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Table 1. Growth comparison between the acetolactate synthase-resistant (*Res*) and three susceptible (*Sus*-1, *Sus*-2, *Sus*-3) biotypes of yellow nutsedge.[a]

Biotype	Tuber density	Dry weight		
		Tuber	Shoot	Root
	No. plant^{-1}	g		
Sus-1	1,706 a	249 b	386 a	761.4 a
	(176)	(11.5)	(43.8)	(71.3)
Sus-2	1,248 b	405 a	269.2 a	692 a
	(49)	(52.7)	(37.1)	(73)
Sus-3	1,231 b	391.2 a	343.7 a	669 a
	(125)	(23.8)	(19.3)	(76.4)
Res	825 c	153.6 c	353.3 a	655 a
	(48)	(16.4)	(34)	(57.1)

[a] Observations were made at 150 d after transplanting, when grown in pots under limited space. Values in parentheses indicate standard errors of mean. Within each column, mean values followed by different letters indicate significant differences ($\alpha = 0.05$).

Mitochondrial nad4 Gene Intergenic Spacer Amplification and Sequencing. Mitochondrial DNA (mt DNA) extraction and PCR reactions were performed as mentioned earlier in the ITS-PCR section. A set of universal primers (Demesure et al. 1995) was used to amplify an intron region of *nad4* gene between exon 1 (5′-CAGTGGGTTGGTTCTGGTATG-3′) and exon 2 (5′-TCATATGGGCTACTGAGGAG-3′). The thermocycler was programed for 4 min initial denaturation at 94 C and 30 cycles of 45 s at 92 C, 45 s at 53.5, 2 min at 72 C, and 10 min final elongation at 72 C (Demesure et al. 1995). Amplicons of ITS-PCR and *nad4* gene were fractioned by electrophoresis in 1.5% agarose gel alongside a GeneRuler^{TM} 100 bp DNA Ladder (Fermentas, provided by the Fisher Scientific–USA, Pittsburgh, PA). Gels were stained in ethidium bromide and imaged using GENi2 gel documentation system. The PCR products were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands; http://www.qiagen.com) and sent for sequencing at the DNA Resource Facility, University of Arkansas. Sequences of ITS-PCR and *nad4* gene-PCR were aligned separately using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2) and trimmed using BioEdit sequencing alignment editor. Molecular evolutionary analysis and phylogenetic tree construction was conducted with bootstrap neighbor-joining method using MEGA version 4 (Tamura et al. 2007).

Results and Discussion

Growth and Reproduction. Under competition for space, differences were observed between the biotypes within the limited growth space, especially for tuber production. Aboveground biomass production at 150 DAT was comparable between the *Res* and *Sus* biotypes (Table 1). Similarly for the belowground biomass, excluding tuber production, there were no differences between *Res* and *Sus* yellow nutsedge biotypes. Overall, the *Res* biotype appears to be identical to *Sus* biotypes in a spreading growth habit under competition for space. Tuber production by the *Res* biotype (total tuber number and tuber weight) was less than the *Sus* biotypes. This can be due to a tendency for the *Res* biotype to produce longer rhizomes and more tertiary daughter plants to aggressively colonize habitats. Bagavathiannan et al. (2015) showed greater overall production of biomass in the *Res* biotype under ample space and greater potential for rapid spread relative to *Sus* biotypes.

Response to Photoperiod. *Aboveground Biomass Production*. There were no significant biotype by photoperiod interactions in aboveground biomass production. However, long photoperiods of 14 and 16 h resulted in greater aboveground biomass than the 12-h photoperiod, irrespective of the biotype (Figure 1). Aboveground biomass averaged across photoperiods differed among biotypes (Figure 2). The *Sus*-1 and *Res* biotypes produced comparable amounts of biomass, whereas biomass production of the *Res* biotype was greater than *Sus*-2 and *Sus*-3 biotypes, showing the existence of natural variation in biomass production among the biotypes.

Tuber Production. Tuber production under the three evaluated photoperiods differed by biotype (Figure 3). The *Res* and *Sus*-3 biotypes produced fewer tubers under a 12-h than 14-h photoperiod, whereas tuber production by the *Sus*-1 was markedly less in the 16 h than in the 12-h and

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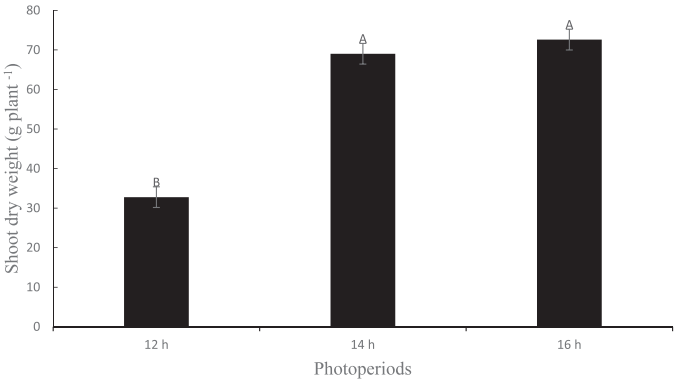

Figure 1. Aboveground biomass production (g) of yellow nutsedge in response to photoperiod, averaged over biotypes (*Res*, *Sus*-1, *Sus*-2, *Sus*-3). No biotype by photoperiod interaction was observed. Different letters indicate significant differences ($\alpha = 0.05$).

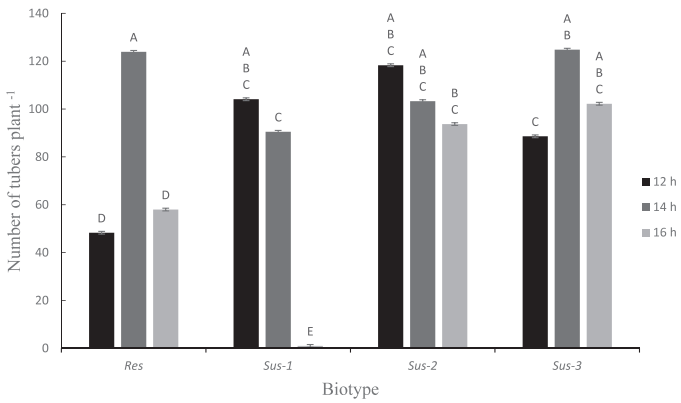

Figure 3. Tuber production of acetolactate synthase-resistant yellow nutsedge (*Res*) and three susceptible biotypes (*Sus*-1, *Sus*-2, *Sus*-3) under three photoperiods. Mean values followed by different letters indicate significant differences ($\alpha = 0.05$).

14-h photoperiods. Each of the *Sus* biotypes produced more than 85 tubers over the 12-wk period regardless of photoperiod, with exception of the *Sus*-1 biotype in the 16-h photoperiod where only two tubers were produced. For the *Res* biotype, tuber production was greater under a 14-h photoperiod than either a 12-h or 16-h photoperiod. These results are slightly different from those of Jansen (1971), who reported a reduction in tuber production at photoperiods longer than 12 h.

Flowering and Seed Production. Similar to previous observations (Bagavathiannan et al. 2015), the *Res* biotype flowered during the 12-wk period under each of the photoperiod treatments. When *Sus* plants from each biotype were harvested, no floral structures were observed and none of these plants formed involucral leaves and axillary inflorescence. The weedy stands of yellow nutsedge also showed an erect growth habit and long rhizomes. Jansen (1971) stated that flowering in yellow nutsedge is influenced only by photoperiod. On the contrary, Mulligan and Junkins (1976) believed that high temperature is the main factor causing flower initiation in yellow nutsedge. In Arkansas, based on our observations, yellow nutsedge plants usually flower from late July to mid-September when temperature reaches 35 C. Two of the three *Sus* biotypes examined here were collected from Arkansas production fields infested by flowering yellow nutsedge. Lack of flowering in these biotypes might result from the artificial growth conditions and maximum temperature of 25 C or factors other than these examined here, such as light quality. Flowering of the *Res* biotype under all photoperiod treatments in comparison with *Sus* biotypes suggests that its photoperiodic flexibility in flowering which can be an important factor for seed production under field conditions.

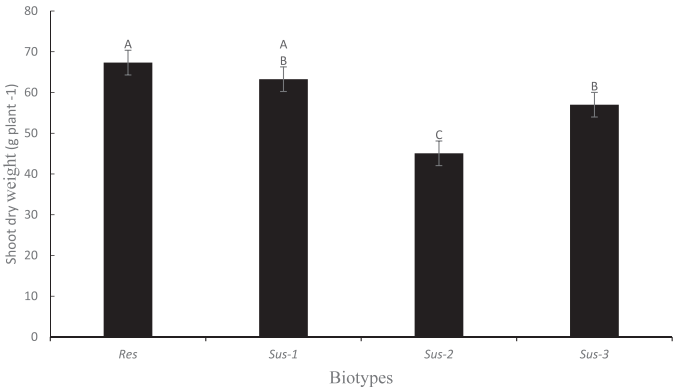

Figure 2. Aboveground biomass production (g) of yellow nutsedge biotypes, averaged over photoperiod treatments. No biotype by photoperiod interaction was observed. Similar letters indicate differences were not significant ($\alpha = 0.05$).

Inflorescence formation and seed production of the *Res* biotype were greatest in the 14-h photoperiod (Table 2). Several *Res* plants did not flower under the 12- and 16-h photoperiods. Tuber production by the *Res* biotype was not reduced by flowering and seed production (Table 2), with maximum tuber and seed production both occurring at the 14-h photoperiod. These results did not confirm the findings of Jansen (1971), who reported that increased flowering reduces tuber production in yellow nutsedge. Irrespective of herbicide resistance, these results and observations demonstrate the existence of wide genetic variation in these biotypes. Phenological plasticity based on quantitative traits (e.g., date of emergence, date of first flowering, and plant height) have been reported

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Table 2. Flowering, seed and tuber production of acetolactate synthase-resistant biotype in response to photoperiod.[a]

Photoperiod	Tuber production	Umbel formation	Seed production
	No. plant^{-1}		
12-h	48.3 b	0.6 b	1.5 b
	(2.19)	(0.24)	(0.37)
14-h	124.9 a	11.4 a	142.8 a
	(3.5)	(1.06)	(3.77)
16-h	58 b	1.6 b	15.5 b
	(2.4)	(0.4)	(1.24)

[a] Values in parentheses indicate standard errors of mean. Within each column, means values followed by the same letters were not significant ($\alpha = 0.05$).

for yellow nutsedge biotypes in California (Holt 1994; Okoli et al. 1997).

The ability of the *Res* biotype to emerge from seed would likely increase its potential for dispersal. Of the seeds sown in pots, seedling emergence was 18% for the 14- and 16-h photoperiods at 72 d after sowing. All emerged seedlings grew well under greenhouse conditions and produced mature plants and tubers. Yellow nutsedge seedling emergence has been rarely recorded in the agricultural fields (Holm et al. 1991). Although tubers are the main cause of yellow nutsedge distribution, herbicide resistance in weed species can be transferred to other fields via pollen (Bagavathiannan and Norsworthy 2014; Burke et al. 2007; Sosnoskie et al. 2012) or seeds. Accordingly, Thullen and Keeley (1979) stated that viable seed production is a potential source of yellow nutsedge regeneration. Furthermore, irrigation significantly increased yellow nutsedge seedling emergence and its survival in field experiments (Lapham and Drennan 1990). The moist soil conditions commonly associated with rice culture in Arkansas would likely aid germination and establishment of this weed from seed, albeit flooding effects on yellow nutsedge seed germination are not known. Another fact that must be considered is that clomazone is the most widely used PRE herbicide in Arkansas rice today (Norsworthy et al. 2013). Clomazone has no activity on sedges, which could further aid establishment of the *Res* biotype. Therefore, it can be concluded that an established *Res* yellow nutsedge seedling can produce tubers in the same growing season and regenerate primarily asexually afterward. Future studies on seedling emergence and survival under field conditions are needed to determine the true likelihood of this biotype to spread by seed.

Sequencing Analysis. *ITS Sequence.* The partial ITS sequences of 600 to 610 bases containing internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2 were obtained using ITS4 primer. Sequences were trimmed to 577 to 581 bp and blasted into the National Center for Biotechnology Information (NCBI) GeneBank. All yellow nutsedge sequences (*Sus*-1, *Sus*-2, *Sus*-3, and *Res*) were 99% identical to *Cyperus esculentus* and the purple nutsedge sequence was 96% similar to *Cyperus rotundus* based on the reference GeneBank sequences. ClustalW2 alignment showed minor nucleotide differences among yellow nutsedge sequences. A phylogenetic tree generated in MEGA 4 using neighbor-joining method showed four main clusters (Figure 4). Among *Sus* yellow nutsdege biotypes, *Sus*-2 and *Sus*-3 clustered together, with 86% bootstrap values apart from *Sus*-1. The *Res* biotype clustered in a separate clade. The greatest distance was observed between yellow nutsedge sequences and purple nutsedge as an outgroup.

ITS-PCR sequence analysis has been widely used in phylogeographical studies to differentiate genera and even genus in plants and fungi (Baldwin 1992; White et al. 1990). This specific region is < 1 kilobase pairs (kbp) in length and consist of relatively conserved noncoding sequences that mutate randomly and demonstrate a greater range of variations than ribosomal DNA (rDNA) genes (McLain et al. 1995; Ritland et al. 1993). According to Hsiao et al. (1999), even minor variations in the ITS region might reveal differences between species and subspecies. High similarity among the ITS sequences of the yellow nutsedge biotypes evaluated here suggest that these biotypes are closely associated and they belong to the same species with different genotypes. On the other hand, minor genetic dissimilarity between the biotypes appears to show some degree of sexual reproduction through seeds. Although it has been shown that somatic mutagenesis can lead to herbicide resistance (Michel et al. 2004) and might occur in tubers and lead to

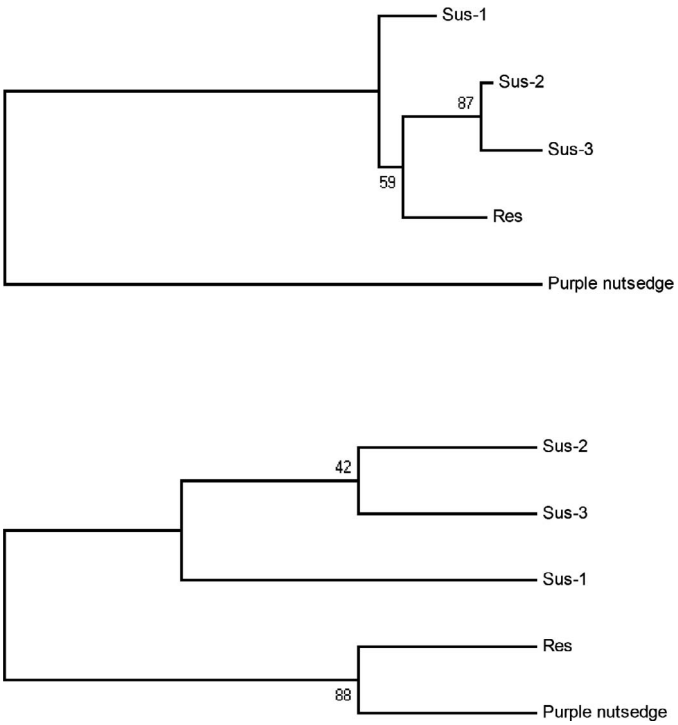

Figure 4. Bootstrap consensus phylogenetic tree of partial ITS (top) and *nad4* gene intron (bottom) sequences of yellow nutsedge biotypes and outgroup purple nutsedge using Neighbor-Joining analysis. Bootstrap values (%) on branching nodes are from 1,000 bootstrap replicates.

some level of genetic dissimilarity (Christian et al. 2013), the levels observed in the present analysis certainly suggest the likelihood of sexual reproduction in the population makeup.

Mitochondrial nad4 Gene Sequence. The NCBI/blast search based on 896 bases of the intron sequences (between exon 1 and exon 2) of the mitochondrial *nad4* gene from all biotypes used in this study exhibited 95% similarity to the mitochondrial genome of different plant species. Based on the sequence alignment and identity matrix created by ClustalW2 (not shown), only few nucleotide variations were observed between the biotypes, and the polymorphism detected between sequences were single nucleotide substitutions (SNPs). Among the three *Sus* yellow nutsedge sequences, *Sus*-2 and *Sus*-3 were 100% identical for the *nad4* gene sequence, whereas *Sus*-1 was > 99% identical to *Sus*-2 and *Sus*-3. In contrast, the sequence of the *Res* biotype was 100% identical to purple nutsedge with > 99% nucleotide similarity to the susceptible biotypes. Neighbor-joining analysis in MEGA4 created a bootstrapped phylogenetic tree with two main clusters with 90% bootstrap values (Figure 4). All *Sus* biotypes were grouped together and *Sus*-2 and *Sus*-3 were in a sub-clade with < 70% bootstrap values. Purple nutsedge and the *Res* biotype sequences were clustered together and grouped away from the *Sus* biotypes.

Cytoplasmic DNA has been of interest in phylogeny, phylogeography, intraspecific hybridization, and population genetics (Demesure et al. 1995; Marshall et al. 2011; Skuza et al. 2007). PCR-based markers are very useful tools for differentiating populations of higher plants at low taxonomical levels that have variable gene arrangement within and between the mitochondrial genome (Schuster and Brennicke 1994). mtDNA has a very conserved sequence (e.g., the *nad4* gene) consisting of intragenic spacers that are mostly inherited through maternal lineage (Duminil et al. 2002; Marienfeld and Newton 1994). Hetroplasmy in the mitochondrial transcriptional unit (e.g., *nad3-orfa56*) of wheat (*Triticum aestivum* L.) × cereal rye (*Secale cereale* L.) hybrid progeny demonstrated potential paternal transmission of the mtDNA molecule (Hattori et al. 2002). Given the distinct genetic grouping of the *Res* biotype based on nuclear and cytoplasmic DNA, coupled with its intermediary growth habit between yellow and purple nutsedge (Bagavathiannan et al. 2015), it is most likely that the *Res* biotype results from hybridization between yellow and purple nutsedge. Interspecific natural hybridization within the genus *Cyperus* has been reported worldwide (Koyama 1961; Manhart 1989). According to Chozin and Yasuda (1991), intermediate morphological traits (e.g., inflorescence structure and density), were observed in F1 progenies of rice flatsedge (*Cyperus iria* L.) and lesser rice-field flatsedge (*Cyperus microiria* Steud.) plants in experimental fields in Okayama, Japan. Furthermore, possible hybridization between yellow and purple nutsedge under field conditions has been reported in California (Tayyar and Holt 2003). These authors reported a novel nutsedge ecotype (CK) similar to purple nutsedge in morphology from an agricultural field near Bakersfield, CA. The CK differed from standard purple and yellow nutsedge plants based on RAPD DNA fingerprinting and phenological analysis, and exhibited a hybrid pattern in the isozyme profile. Multimarker PCR-based analysis (Carstens and Knowles 2007) to screen large portions of mtDNA, or whole mitochondrial or chloroplast genome sequencing through next generation sequencing technology will provide more information on the diversification of yellow nutsedge.

Overall, molecular analysis along with in vivo experiments suggest that the *Res* biotype is

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genetically different from the *Sus* biotypes. Based on the findings from the present study and those of Bagavathiannan et al. (2015) who showed that the *Res* biotype mimics patterns of spread in purple nutsedge, it can be concluded that the *Res* biotype is likely a hybrid between yellow and purple nutsedge.

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