JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Management on the Yield and High-Molecular-Weight Polysaccharide Content of Shiitake (*Lentinula edodes*) Mushrooms

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Shiitake (*Lentinula edodes* (Berk.) Pegler) mushroom production in the United States has increased greatly over the last twenty years. Additional expansion of the shiitake mushroom market should be possible if the product can be marketed as a functional food, i.e., a food that has health-promoting effects beyond its nutritional value. High-molecular-weight polysaccharides (HMWP) including lentinan in shiitake may promote human health. This study was conducted to determine if management protocols influence the HMWP of shiitake mushrooms. Results indicate that measuring the total carbohydrate content of water-extractable, ethanol-insoluble polysaccharides was a simple way to estimate HMWP. Results also indicate that log-grown shiitake contained more HMWP than did substrate-grown shiitake. Among log-grown shiitake, both mushroom strain and tree species influenced HMWP content. The results suggest that there is considerable variation among shiitake mushrooms in HMWP content and that production protocols influenced the HMWP content of mushrooms.

KEYWORDS: Lentinan; log-grown; substrate-grown; strain; tree species; carbohydrates; growth conditions; shiitake mushroom; *Lentinula edodes*

INTRODUCTION

Shiitake (*Lentinula edodes* (Berk.) Pegler) mushroom production in the United States has expanded greatly since its start 25 to 30 years ago. Between 1990 and 1995, shiitake production doubled to over 2.5 million kilograms annually (*1*). Production has continued to increase since then. Current production exceeds 4,000,000 kg. Today, the industry can readily be segregated into two different types of production systems. One system utilizes an artificial substrate to produce mushrooms. This production system accounts for a large portion of the total shiitake production in the United States and yields mushrooms that are sold fresh in bulk at relatively low prices in grocery stores. The other production system utilizes natural logs to grow mushrooms. Log-grown mushrooms tend to be organically grown and are marketed either fresh to high-end users such as restaurant chefs or dried as a component of a partially prepared organic food. Prices for log-grown shiitake tend to be 3 to 8 times higher than that of substrate-grown shiitake, making them attractive for small farmers or woodlot owners interested in a relatively high return on their management. A higher return is necessary because the production of log-grown mushrooms is fairly labor intensive, especially compared to production of mushrooms on substrate. However, at present the markets for log-grown mushrooms are limited. In addition, not all producers have the skills to deliver a very-high-quality end product to consumer groups, like restaurant chefs, who tend to have demanding product specifications.

It may be possible to increase the demand and markets for log-grown shiitake if these mushrooms can be promoted as a functional food, i.e., a food that has value beyond its nutritional value because it has unique properties that promote specific aspects of human health. Shiitake mushrooms have had such a reputation in Asian countries for centuries (*2*, *3*). If these claims can be scientifically substantiated, and if these properties can be optimized in cultivation, it may be possible to effectively promote log-grown shiitake as a functional food to American consumers.

Many health benefits have been ascribed to shiitake mushrooms and compounds isolated from them. There is substantial evidence that consumption of shiitake mushrooms will decrease serum cholesterol (*4*). The primary compound in shiitake responsible for lowering serum cholesterol is eritadenine, a secondary metabolite with structural characteristics similar to those of adenine (*5*). Eritadenine decreases serum cholesterol by accelerating the metabolism of cholesterol in the liver (*4*). In addition to the effects of eritadenine, ingestion of crude fiber from shiitake also lowers serum cholesterol (*6*). Crude fiber, in general, lowers serum cholesterol by reducing absorption during digestion (*7*), and water-soluble fiber is relatively abundant in shiitake mushrooms (*6*, *8*).

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10.1021/jf020080l CCC: $22.00
Published on Web 08/03/2002

Both the homogeneous and heterogeneous polysaccharides/glucans and glucan−protein complexes from fungi have been identified as promoting health in humans (*9*). Shiitake mushrooms contains a water-soluble homogeneous polysaccharide/β-glucan, lentinan, that has been identified as health promoting (*2*, *3*). Shiitake may contain other glucans and glucan−protein complexes with health-promoting properties, but such constituents have not been identified to date. Therefore, lentinan is an obvious choice to study if one is to focus on glucans as a way of promoting shiitakes as a functional food. Lentinan consists of a β(1−3) linked D-glucan backbone with two β(1−6) linked glucose side chains for every five glucose residues and has a molecular weight in excess of 400,000 Da (*10*). Lentinan is readily soluble in water but insoluble in 50% (v/v) ethanol (*11*, *12*). There is abundant evidence that intravenous injections of lentinan can promote human health by stimulating the immune system, and this stimulation has been used to treat various forms of cancers and AIDS in countries outside of the United States (*13*). Ingestion of lentinan may also promote a healthy immune system (*14*); however, the health effects of lentinan ingestion have not been studied as extensively as response to lentinan injections.

There are few data in the literature on the content of β-glucans and lentinan in shiitake mushrooms. Mizono et al. (*12*) reported the development of an ELISA assay to detect levels of lentinan in mushrooms. They reported that shiitake mushrooms contained 3.4 mg lentinan g^{-1} (fresh weight), whereas mushrooms of two *Agaricus* species lacked lentinan. Mushrooms from the genera of *Flammulina*, *Sarcodon*, *Meripilus*, *Panellus*, *Lactarius*, *Hygrophorus*, and *Grifola* had lower levels of lentinan than shiitakes. Manzi and Pizzoferrato (*15*) adapted a protocol for the quantitation of total β-glucans from cereals for use with edible mushrooms and found that edible mushrooms differed considerably in their β-glucan content. Results of Mizono et al. (*12*) and Manzi and Pizzoferrato (*15*) indicate that considerable variation in lentinan/β-glucan content exists among mushrooms. One remaining question is whether management protocols to produce edible mushrooms affect the content of these polysaccharides. The objectives of this study were (1) to find a relatively simple and fast method to quantitate water-soluble, high-molecular-weight polysaccharides (HMWP), which would include lentinan; and (2) to use the assay to assess the effects of management on the HMWP content of shiitake mushrooms.

MATERIALS AND METHODS

Development of Assay for HMWP. Total carbohydrates were analyzed according to the protocol of Brink et al. (*16*) using a high-molecular-weight dextran as the standard. HMWP were extracted with hot water. Mushrooms (2 g) were suspended in 20−30 mL of 40° C highly purified water. This suspension was incubated for 15 to 18 h at room temperature (18−22° C) on a rotary shaker moving at 150 rpm. The suspension was filtered through cheesecloth. Highly purified water (5 mL, 40 °C) was used to rinse the flask and the residue on the cheesecloth, and combined with the initial cheesecloth filtrate. The combined filtrate was centrifuged at 12 000*g* for 30 min. at 15° C. This supernatant was referred to as the crude extract. A 10-mL aliquot of the crude extract was combined with 10 mL of ethanol in another centrifuge tube. After incubating for 10 min, the ethanol−water suspension was centrifuged at 12 000*g* for 30 min at 15° C. The resulting pellet was dissolved in 10 mL of 40° C highly purified water to yield the resuspended ethanol precipitate. Initially, 0.75 mL of the resuspended ethanol precipitate was subjected to fractionation by size-exclusion chromatography on a 1 × 7 cm column of Sepharcyl S-300. The column had been equilibrated and eluted with a buffer containing 20 mM Na−HEPES (pH 7), 50 mM NaCl, and 5 mM NaCN. The volume corresponding to the void volume, as determined by the elution of blue dextran, was collected, and was initially referred to as the HMWP fraction. In certain experiments, noted in the text, 0.75 mL of crude extract was fractionated by size-exclusion chromatography on a S-300 column as described. In later experiments, carbohydrates in the resuspended ethanol precipitate were subjected to fractionation on a Sepharcyl S-500 column (1 × 28 cm) equilibrated and eluted with the buffer described above. No differences in total carbohydrate content of the crude extract or the fractions resulting from ethanol precipitation or size-exclusion chromatography were found when extractions as prepared above were compared to those prepared at 40° C overnight (data not shown).

Table 1. Time of the Four Forced-Flushing Periods and Air Temperature during These Time Intervals for Expts 1 and 2[a]

flushing		temperature, °C		
period	dates	minimum	maximum	average daily mean
autumn 1999	Nov. 1–8, 1999	–1.1	26.1	15.6
spring 2000	April 27–May 4, 2000	8.9	26.7	17.7
autumn 2000	Oct. 17–26, 2000	5.6	27.8	18.3
spring 2001	April 5–15, 2001	10.0	31.1	21.7

[a] Daily mean temperature is the average between the daily high and low. The average daily mean temperature is the average of the daily mean temperatures throughout the flushing period.

The above protocol was tested using three commercially available preparations of HMWP from shiitake differing in lentinan purity and content. Purified lentinan (batch number 990303), *Lentinula edodes* mycelium extract, and shiitake mushroom extract, 10% polysaccharide, were obtained from Sihai Plant Extract Co., Ltd. of Japan. The recovery of carbohydrates from these preparations was compared.

Experiments 1 and 2. For experiments 1 and 2, logs of southern red oak (*Quercus falcata* Michx. var. flacata) approximately 1.3 m long and 10−15 cm in diameter were inoculated with sawdust containing one of three different strains of shiitake mushrooms (*Lentinula edodes*) in the winter of 1998 and 1999 essentially as described previously (*17*, *18*). Logs were stored under ambient conditions in the shade in Shirley, AR until they were transferred to the Dale Bumper Small Farm Research Center south of Booneville, AR in October 1999. Once in Booneville, logs were stacked nearly upright with one cut edge on the ground under the shade of 4−5 m tall loblolly pine (*Pinus taeda* L.) trees. Logs were occasionally sprayed with water to prevent excessive moisture losses, especially during the hot, dry months of August and September. Logs were submerged in cold water for 24 h to force-flush (i.e., encourage the production of mushrooms) in the fall and spring of each year (**Table 1**). After submersion, logs were arranged in a completely randomized block design with 10 replications. Each log was treated as a replicate. Mushrooms were collected daily up to 10 days following submersion. The fresh weight and number of mushrooms were recorded per log for each of four flushing events. After the mushrooms were weighed, they were frozen at −20° C, lyophilized, and stored at −20 °C until analyzed for HMWP content.

The objective of expt 1 was to compare the HMWP content of mushrooms from artificial substrate to that of log-grown mushrooms. In the autumn of 2000 and spring of 2001, blocks of artificial substrate using an oak sawdust as the base material were soaked with water to induce mushroom production, and were incubated outside alongside the logs mentioned above. These blocks were obtained from Fox Valley Farms (Lyons, OR) and handled according to the vendor's instructions. Mushrooms produced from these artificial substrate blocks were collected, counted, and weighed. Ten samples representing a composite of the mushrooms produced by the red oak logs described above were collected and handled in a manner similar to that of mushrooms produced on the artificial substrate blocks. Additionally, three packages of substrate-grown shiitake were purchased from a local grocery store on two different occasions and handled in the same manner as log- and substrate-grown mushrooms. Mushrooms were frozen at −20° C soon after harvesting. Mushrooms were lyophilized, and the dried mushrooms were stored at −20° C until analyzed for HMWP.

Table 2. Recovery of Carbohydrates from Three Commercially Available Preparations from Shiitake Mycelium, Mushrooms, and Purified Lentinan Subjected to Ethanol Precipitation and Size-Exclusion Chromatography[a]

	% of carbohydrate recovered		
sample	size-exclusion chromatography	ethanol precipitation	ethanol precipitation and chromatography
shiitake extract	79 ± 3	22 ± 3	22 ± 2
LEM[b]	20 ± 4	5 ± 2	5 ± 2
purified lentinan	103 ± 2	106 ± 2	99 ± 3

[a] Aqueous suspensions of the three preparations were assayed for total carbohydrates and then subjected to precipitation by 50% (v/v) ethanol, size-exclusion chromatography on S-300 gel matrix, or ethanol precipitation followed by size-exclusion chromatography. The resulting fractions were then analyzed for total carbohydrates and compared to the original aqueous suspension. Each entry is the average of 9 observations plus or minus SE. [b] LEM, *Lentinula edodes* mycelium extract.

The objective of expt 2 was to determine effects of years after inoculation, season, and shiitake strain on mushroom yield and HMWP content in response to force flushing. The objective of this experiment was to estimate typical mushroom yields induced by force flushing, not to account for total mushroom production by the logs over the 2 years. Air temperature data immediately before and following submersion were collected from the weather station located near the Petit Jean River just south of Booneville, AR, approximately 5 miles east of where the mushrooms were grown (**Table 1**). Data from expt 2 were analyzed as a completely randomized design with 10 replications by analysis of variance using the SuperANOVA program (Abacus Concepts, Inc., Piscataway, NJ, 1991, version 1.11). Data are reported as means plus or minus the standard error (SE).

Experiment 3. The objective of expt 3 was to determine the effects of tree species and shiitake strain on HMWP content. Logs from sweet gum (*Liquidambar styraciflua* L.), white oak (*Quercus alba* L.), and southern red oak (*Quercus falcata* Michx. var. flacata) approximately 1.3 m long and 10−15 cm in diameter were inoculated with sawdust containing one of two different strains of shiitake mushrooms (*Lentinula edodes*) in the winter of 1998 and 1999 essentially as described previously (*17*, *18*). Logs were stored under ambient conditions in the shade in Shirley, AR, until they were flushed in the spring of 2001. Logs were submerged in cold water for 24 h to force a flushing. Logs were then incubated in a room where temperature and relative humidity were maintained at 20 °C and 50−70%, respectively. Logs representing replications were arranged within the room as a completely randomized design with 12 logs/replication. Mushrooms were collected from logs over a 1-week interval, frozen at −20 °C, lyophilized, and stored at −20 °C until analyzed for HMWP content. Data were analyzed by analysis of variance using the SuperANOVA program as a completely randomized design with logs serving as replications. Data are reported as means plus or minus the standard error (SE).

RESULTS AND DISCUSSION

Development of a Protocol to Estimate HMWP Content. The objective of this portion of the project was to develop a relatively simple protocol to estimate the content of HMWP that would include lentinan. Aqueous preparations from the three commercial preparations were subjected to size-exclusion chromatography on S-300 gel filtration matrix, which excludes dextrans in excess of 300,000 Da. All of the carbohydrate in the purified lentinan was recovered in the void volume of such columns (**Table 2**). Only 20% of the carbohydrate from the mycelium extract was recovered in the void volume of the S-300 column, indicating that polysaccharides were only a small fraction of this preparation. Almost 80% of the carbohydrates in the shiitake extract were recovered in the void volume, indicating that a majority of the polysaccharides in this preparation were HWMP.

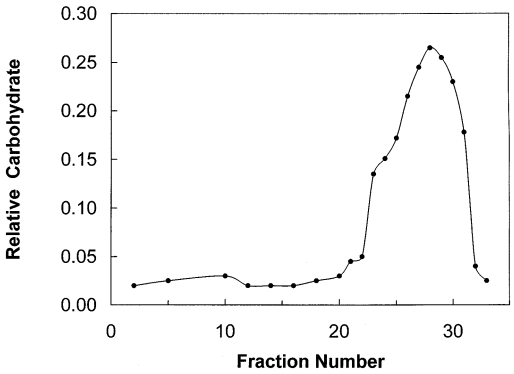

Figure 1. Separation of carbohydrates from shiitake mushrooms by size-exclusion chromatography on S-500 matrix. Carbohydrates were water extracted and then precipitated by 50% (v/v) ethanol prior to fractionation on the S-500 column. The mean elution volume of blue dextran was at fraction 11 and the internal volume corresponded to fraction 33.

All of the carbohydrates in the lentinan preparation were recovered when subjected to precipitation by 50% (v/v) ethanol. Significantly less carbohydrate was recovered from the shiitake and mycelium extracts by ethanol precipitation than by size-exclusion chromatography (**Table 2**). These results indicate that precipitation by ethanol was more selective than size-exclusion chromatography for the recovery of HMWP. The amount of carbohydrates in the original extract recovered in the void volume of S-300 after ethanol precipitation was approximately the same as that of ethanol precipitation alone. The recovery of carbohydrate after size-exclusion chromatography followed by ethanol precipitation from the three commercial preparations was approximately the same as that achieved from ethanol precipitation alone, and that of ethanol precipitation followed by size-exclusion chromatography (data not shown). Taken together, these results indicate the simplest means of estimating HWMP content that would include lentinan would be to subject an aqueous extract to ethanol precipitation and assay the precipitate for total carbohydrates. Such a protocol was employed in the experiments described below. The protocol described above is simpler than those described previously (*12*, *15*), because this procedure does not use enzymes or antibodies which can have limited shelf life and require more care in use. Shiitake mushrooms have not been analyzed by the three different methods. Therefore, comparisons of content data among the different methods probably are of limited value.

To define the distribution of molecular weights of the water-extractable carbohydrates from shiitake mushrooms, the ethanol-insoluble component was fractionated on an S-500 column (**Figure 1**). The S-500 matrix separates dextrans with molecular weights between 40,000,000 and 40,000 Da. Most of the water-extracted, ethanol-insoluble carbohydrates eluted from the S-500 as a fairly symmetrical peak with a slight shoulder toward the high molecular component (i.e., lower fraction number) with a retention slightly greater than that of blue dextran. The elution pattern indicates that most of the carbohydrates had molecular weights between 2,000,000 and 40,000 Da, within the range of that reported for lentinan (*13*).

Experiment 1. For mushrooms produced during the autumn of 2000 and spring of 2001, the HMWP content of log-grown mushroom exceeded that of either source of substrate-grown mushroom by at least 70% (**Table 3**). The HMWP content of store-bought substrate-grown mushrooms was the lowest. The relatively low content of such mushrooms may result from autolysis of HMWP during storage and transport as observed previously (*11*).

Table 3. High-Molecular Weight Polysaccharide (HMWP) Content of Log-Grown and Substrate-Grown Shiitake Mushrooms

type of mushroom	average HMWP content mg g^{-1} (d.w.)[a]	SE	*n*
log-grown	38.6	2.4	20
substrate grown	22.4	1.5	12
store bought/substrate grown	13.7	0.7	6

[a] Abbreviations: SE, standard error of the mean; *n*, number of observations; d.w., dry weight.

Table 4. Effects of Years, Seasons, and Shiitake Strains on the Production of Mushrooms Growing on Oak Logs and Their High-Molecular-Weight Polysaccharide Content (HMWP)[a]

		mushroom production per log				HMWP	
		total mass (g)		number		content	
source of variation	df[b]	*F*-test		*F*-test		*F*-test	
years	1	1.8	ns	0.7	ns	0.3	ns
seasons	1	16.0	***	20.9	***	0.5	ns
years × seasons	1	6.9	**	9.6	**	0.6	ns
strain	2	6.0	**	13.7	***	3.9	*
strain × years	2	9.6	***	13.6	***	1.3	ns
strain × seasons	2	0.9	ns	1.6	ns	0.3	ns
strain × years × seasons	2	11.8	***	11.0	***	0.5	ns

[a] Results are from expt 2. [b] Abbreviations: df, degrees of freedom; ns, *, **, *** denotes *F*-test is not significant at $P = 0.05$, significant at $P = 0.05$, 0.01, or 0.001 levels of probability, respectively.

Experiment 2. The effects of shiitake strain on the growth of shiitake mushrooms inoculated into oak logs in response to flushing and their HMWP content was measured in the fall and spring over a 2-year period (**Table 4**). Analysis of variance indicated that seasons and strains affected mushroom production per log per flushing, but that year did not (**Table 4**). Trends were similar between the two yield components: fresh weight produced per log and number of mushrooms produced per log. Therefore, only data for g (fresh weight) of mushrooms produced per flushing per log are reported. On average, mushrooms weighed 10 g (fresh weight) independent of either flushing or strain (data not shown). Across seasons and years, Westwind produced more mushrooms, averaging 102 ± 12.7 g per log per flushing, than Coldweather or Snowcap, which averaged 56 ± 12.5 and 50.1 ± 8.5 g per log per flushing, respectively. Fall flushings induced a greater production of mushrooms than spring flushings: 92.3 ± 12.5 g versus 36.5 + 7.2 g per log. There were significant interactions for the year × season, strain × year, and strain × year × season sources of variation (**Table 4**). The basis for these interactions is apparent from **Figure 2**. Mushroom production by logs inoculated with Westwind peaked in the fall of year two (11 months into the study), whereas production by logs inoculated with either Coldweather or Snowcap declined throughout the study. There was no difference in the time course of mushroom production by logs inoculated in 1998 as compared to those inoculated in 1999 (data not shown).

Strain had a significant effect on HMWP content but there were no significant effects of season or season × strain interaction (**Table 4**). Mushroom production and HMWP content data were gathered at a time of year when daily mean temperatures were similar (**Table 1**). Larger differences between strains and a significant season × strain interaction may have been detected if data were gathered at times of the year when the temperatures were more favorable for the cold-weather strains. In this experiment the HMWP content of Westwind averaged 39.6 ± 2.4 mg g^{-1} (dry weight), which was greater than that of Snow Cap and Cold Weather which averaged 31.1 ± 2.1 and 32.8 ± 1.8 mg g^{-1} (dry weight), respectively.

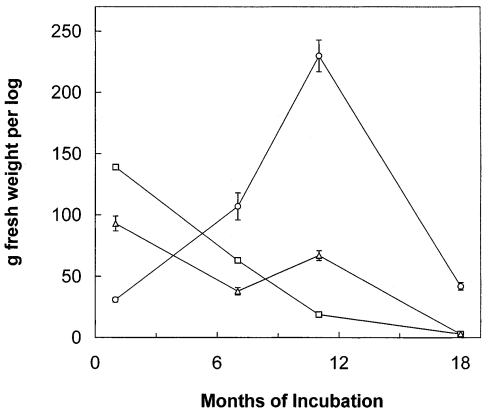

Figure 2. Mushroom production per log after flushing over a two-year period. Data are presented by Shiitake strain: Westwind (○), Snow Cap (△), and Cold Weather (□). Time is depicted as months after establishment of logs at the Booneville location in October, 1999. Data are means with the SE depicted as bars.

Table 5. Summary of the Analysis of Variance Examining the Effects of Shiitake Strain and Tree Species on the High-Molecular-Weight Polysaccharide (HMWP) Content of Log-Grown Shiitake Mushrooms[a]

source of variation	df	*F*-test	significance
tree species	2	4.1	*
strain	1	17.4	**
tree species × strain	2	3.8	*

[a] Results are from expt 3. See **Table 2** for definitions of abbreviations.

Table 6. Effects of Shiitake Strains and Tree Species on the High-Molecular-Weight Polysaccharide (HMWP) Content (mg g^{-1} (dry weight)) of Shiitake Mushrooms[a]

	strain	
tree species	Snow Cap	Westwind
sweet gum	32.2 ± 1.6	61.2 ± 3.1
white oak	61.6 ± 4.8	66.8 ± 7.4
red oak	43.9 ± 4.4	95.8 ± 17.7

[a] Data are means from 12 observations per strain × tree species entry plus or minus SE. Data are from expt 3.

Experiment 3. Mushrooms produced by three species of trees inoculated with either SnowCap or Westwind were analyzed for HMWP content (**Tables 5** and **6**). Both tree species and mushroom strain had a significant effect on HMWP content (**Table 5**). When averaged across tree species, Westwind had a higher content of HMWP than Snow Cap, 74.6 ± 7.8 versus 45.9 ± 4.7 mg g^{-1} (dry weight). Therefore in both of these experiments, mushrooms produced by Westwind had a greater HMWP content than SnowCap. The HMWP content of the mushrooms from this second experiment were greater than that of the previous, suggesting that management decisions other than strain and tree species influence HMWP content. The HMWP content of mushrooms from sweet gum logs was less than that of mushrooms from either red or white oak (Table 6). Averaged across strains, HMWP content of sweet gum logs was 46.7 ± 6.7 mg g-1 (dry weight) as compared to averages of 64.2 ± 4.1 and 69.9 ± 10.1 for white and red oak logs, respectively. The HMWP content of Snowcap mushrooms was greatest when white oak logs were inoculated, while the content

of Westwind mushrooms was greatest when red oak logs were inoculated.

General Discussion. The results of these experiments indicate that log-grown shiitake mushrooms have a greater content of a high molecular polysaccharide fraction than substrate grown mushrooms. These results also indicate that management and growing conditions may manipulate the polysaccharide content of log-grown mushrooms. It should be noted that logs inoculated with Westwind not only yielded a greater quantity of mushrooms but also had higher HMWP content. Therefore through management it may be possible to produce a relatively large quantity of mushrooms with high contents of health promoting polysaccharides (Figure 2, Tables 4 and 6). Future research is needed to more fully understand the effects of production protocols on HMWP.

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Received for review January 24, 2002. Revised manuscript received June 10, 2002. Accepted June 14, 2002. Expenses for part of the research conducted in this article were defrayed by a specific cooperative agreement (58-6227-9-042) between ARS and the Shirley Community Development Corporation.

JF020080L