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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Functional analysis of the UDP glucose: Flavonoid-3-Oglucosyltransferase (UFGT) promoter from litchi (*Litchi chinesis* Sonn.) and transient expression in onions (*Allium cepa* Linn.)

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The expression of the UDP glucose: flavonoid-3-Oglucosyltransferase (UFGT), which encoded the last enzyme of the anthocyanin pathway, was under developmental control as well as affected by external stimuli such as ABA. Three fragments of the 1.38 kb upstream region of the UFGT gene from Litchi (*Litchi chinesis* Sonn.) were fused to the GUS-coding region, and the expression of these constructs was analyzed in onions. To characterize the cis-regulatory functions of the promoters for enzymes in anthocyanin biosynthesis, we examined onions carrying a series of nested UFGT promoter-β-glucuronidase (GUS) fusion for GUS activity by histochemical staining. The AE-box and ELI-box in anthocyanin biosynthesis of angiosperm were found in UFGT promoter. MRE which was MYB binding site involved in light responsiveness, control the transcription of genes in anthocyanin biosynthesis. The region of -910 to -344 in UFGT promoter showed high activities in the three parts. Although the expression characteristics were indistinguishable from those of the full-length promoter, we observed differences in UFGT promoter regulation for the different construct. The results suggested that region of 910 to 344 of UFGT promoter had multiple functions in the expression under the various developmental stages and stress conditions in litchi.

Key words: Flavonoid-3-Oglucosyltransferase (UFGT), promoter, litchi, transient expression.

INTRODUCTION

The litchi (*Litchi chinesis* Sonn.) is a tropical and subtropical fruit tree native to southern China, Taiwan, Bangladesh and Southeast Asia, and now cultivated in many parts of the world. The litchi has a history and cultivation going back as far as 2000 BC according to records in China. Cultivation began in the area of southern

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China, Malaysia and Vietnam. Wild trees still grow in parts of southern China and on Hainan Island. Litchi is extensively grown in China, and also elsewhere in Brazil, South-East Asia, India, Pakistan, Bangladesh, southern Japan and more recently in California, Jamaica and elsewhere in the Caribbean, Hawaii, Texas, Florida, the wetter areas of eastern Australia and sub-tropical regions of South Africa, Israel and also in the states of Sinaloa and San Luis Potosíin Mexico. A wide range of cultivars is available, with early and late maturing forms suited to warmer and cooler climates, respectively. They are also grown as an ornamental tree as well as for their fruit. Litchi was commonly sold fresh in Asian markets, and in recent years, also widely in supermarkets worldwide. The red rind turns dark brown when the fruit is refrigerated, but the taste is not affected. It is also sold canned year-round. Anthocyanin biosynthesis had been characterized in flowers of petunia, snapdragon, pericap of litchi, skins of grape and in kernels of maize, and the biosynthetic pathway is now one of the best known pathway in plants (Holton and Cornish, 1995). Anthocyanin was the predominant pigments of grape skins as well as other blue, red, or black fruits and flowers, and was biosynthesized through a flavonoid pathway. Regulation of the anthocyanin pathway had been shown to occur mainly at the transcriptional level in pigmented organs. Two classes of genes were required for anthocyanin biosynthesis. The structural genes encoded the enzymes that directly participate in the formation and storage of anthocyanins and other flavonoids. The UGFT enzyme of plants was the final gene in the anthocyanin pathway. The transfer of the glucosyl moiety from UDP-glucose to the 3-hydroxyl group of anthocyanidins by UFGT was shown to be the key for anthocyanidin stability and water solubility (Yoshihara et al., 2005). In grapevines, the main control point for anthocyanin quantitative variation was downstream in the pathway at theUFGT level, in agreement with the early observations (Boss et al., 1996a, b, c). In the grape berry, anthocyanin biosynthesis pathway, some cDNAs encoding structural genes had been cloned in *Vitis vinifera* (Sparvoli et al., 1994). It was showed that only the expression of the gene coding for UFGT was consistently associated with the berry color, depending on developmental stage and cultivar (Boss et al., 1996a, b, c). Expression analysis of UFGT genes in white and red-skinned cultivars revealed that the UFGT gene was present, but not expressed in the white cultivars (Boss et al., 1996a, b, c; Kobayashi et al., 2001). The UFGT sequences were identical in white and red-skinned sports including the promoter region, suggesting that the phenotypic change from white to red could be the result of a mutation in a regulatory gene controlling the expression of UFGT (Kobayashi et al., 2002). Commercial litchi cultivation had focused on fruit crops that develop red color within a month time on the tree. Therefore, this fruit could provide a model for investigating regulation of anthocyanin synthesis during fruit maturation. Here, we reported the isolation of the promoter of UFGT gene in the anthocyanin biosynthetic pathway. Expression of the GUS gene was examined in a series of nested UFGT promoter. The evidence presented revealed that GUS activity was the highest in one element of about 360 bp upstream of the transcription start site.

MATERIALS AND METHODS

Plant material and bacterial strains

Litchi (*L. chinesis* Sonn.) cv. Nuomici was grown at College of Horticulture, South China Agricultural University in Guangzhou, China. Fresh leaves and fruit samples were taken to the laboratory and immediately frozen in liquid nitrogen and stored at -80°C. *Escherichia coli* strain DH5a was cultivated in LB medium for vector constructs and DNA manipulation. *Agrobacterium tumefaciens* strain EHA105 was cultivated in LB medium for onions transformation. White skin onion cultivar was used for transformation.

PCR cloning of the UFGT promoter region

Total genomic DNA was isolated from fresh leaves samples by CTAB method (Stewart and Via, 1993). The promoter region was cloned with high-efficiency thermal asymmetric interlaced PCR method described previously (Liu and Chen, 2007). The corresponding produce was subjected to pre-amplification reactions of TAIL-PCR with inner gene-specific primer (GSP1) (5'-TTC CTT CTT TGT TCT CTC AGC AGA CC-3') and the primary or secondary TAIL-PCRs with outer gene-specific primer (GSP2) (5'-ATT GTG TGTAGC AAC AGC AGG GC-3'). Major bands were isolated from using TaKaRa Agarose Gel DNA Purification kit (TaKaRa, Japan), and the isolated fragments were cloned into a pMD-19T vector (TaKaRa, Japan). The inserted sequence in recombinant plasmid DNA was analyzed using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA) on an ABI PRISMTM 377 DNA Sequencer. In order to find out motifs regulation *UFGT* gene expression, functional motifs in *UFGT* gene promoter were predicted by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Construction of vectors

With the genomic as a template, a fragment upstream of the translational start codon of *UFGT* gene and its 5' deletion derivatives were generated by PCR with five primers: (Pf1:5'-CAAGCTTGGCCGCCCTGTTGGTTAAATAGTAT-3'; Pr1:5'-CGGATCCAGTGGTGGTGTTGTGGTGGTGGATA-3'; Pf2:5'-CAAGCTTTTAGTAAGGGCTGGATTAAGTGAAT-3'; Pr2:5'-CGGATCGAGGTTCTTAGTGCGTATCGGTCTTCG -3';Pf3:5'-CAAGCTTGACTGATATTTCGGTTTGAACTGCA-3'; Pr3: 5'-CGGATCCCCCCACGCTCACTACGAGACTACCC-3';), the introduced *Hind*III sites in the forward primers and the introduced *Bam*HI site in the reverse primers. The amplified fragments were respectively inserted into the plasmid pBI121 (Clontech) as a *Hind*III-*Bam*HI fragment at the corresponding restriction sites in place of the cauliflower mosaic virus (CaMV) 35S promoter region, resulting in a series of pBI-pUFGT::GUS vectors.

Onions transformation

Individual binary vectors, including pBI121 and a series of

Figure 1. PCR product of *UFGT* promoter was separated on an agarose gel.

pBI-pUFGT::GUS were introduced into *A. tumefaciens* EHA105 by the freeze-thaw method (Walkerpeach and Velten, 1994).

The fresh onion outside 3 to 4 layers scales was removed , the bulb immersed in 75% ethanol with 10 min, washed three times with sterile water. Onions were cut with sterile scalpel bulbs, corms with fresh and hypertrophy of the internal scales including epidermal (concave) were cut an area of 1-2 cm of the small box. After the pre-incubation of onion tissue, they were placed in MS liquid medium re-suspended in broth for 20 min, slightly drained broth, spread on MS solid medium to photoperiod 16/8 h at 25°C, co-culture. After 16-24 h, the onion small piece of skin was removed with a clean wash liquid MS medium slightly shaken to remove the attached Agrobacterium. Transformed onion tissues were measured with histochemical staining and GUS activity.

Fluorometric quantification of GUS activity and histochemical staining

Plant tissues were ground into a fine powder using liquid nitrogen with a mortar and pestle, and suspended in GUS extraction buffer (50 mM sodium phosphate, pH 7.0; 0.1% Triton X-100; 10 mM 2-mercaptoethanol; 10 mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid and 0.1% sodium lauryl sarcosine). The supernatant was collected after centrifugation at 12 000× *g* for 10 min at 4°C. Fluorometric quantification of GUS activity was performed using 4-methylumbelliferyl-b-D-glucuronide substrate (Jefferson et al., 1987). The content of total proteins was determined using the Bradford (1976) method. The GUS activity was expressed as pmol of 4-methylumbelliferone per mg protein per min. Histochemical localization of GUS activity was performed as follows: samples were fixed with 0.5% paraformaldehyde in 0.1 M sodium phosphate (pH 7.0) for 30 min, then various tissues of transgenic tobacco samples were incubated in 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc) solution at 37°C from 3 h overnight until the blue staining reached sufficient intensity (Grotewold et al., 1994). Photosynthetic tissues were cleared of chlorophyll by passaging through a 70-100% ethanol series. Photography was performed with a camera (Nikon 8700, Japan).

RESULTS AND DISCUSSION

Isolation and characterization of litchi UFGT promoter

With the genomic as a template, a 1487 bp fragment upstream of the translational start codon of *UFGT* gene was gotten (Figure 1). The three expression of vector were named P(-1487/-18), as the full-length promoter construct in this study, P1(-1487/-857), P2(-910/-344) and P3(-477/-18) (Figure 2).

The transcriptional start sites of *UFGT* were determined and located 85 bp upstream from the ATG codon. The AE-box, and ELI-box in anthocyanin biosynthesis of angiosperm were found in UFGT promoter. MRE which was MYB binding site involved in light responsiveness, control the transcription of genes in anthocyanin biosynthesis (Grotewold et al., 1994; Jin et al., 2000; Moyano et al., 1996; Sablowski et al., 1994; Sainz et al., 1997; Sugimoto et al., 2000; Tamagnone et al., 1998; Yang, 2001). The *UFGT* gene promoter contained TCA elements and a HSE homologous sequence, UFGT promoter also contained WUN-motif that was wound-responsive element (Figure 3).

Expression pattern of the UFGT promoter in onions (*Allium cepa* Linn.)

In order to identify promoter regions that confer the spatial and developmental expression of litchi genes, the 1.48 kbp promoter fragment and three promoter deletions of UFGT were fused to a GUS reporter gene (Figure 4). All constructs were transferred into onions plants by Agrobacterium-mediated transformation system. Ten to 15 independent onions transformants were obtained for each construct. Figure 5 showed the GUS activities in onions transformants with the UFGT promoter-GUS construct. The GUS expression levels of the the P1promoter-GUS construct (-1487/-857) and the P3promoter (-477/-18) fragments were very low in onions plants. The highest level of GUS activity was detected in transformants carrying UFGT full length (-1,487 bp from the transcriptional start site). We then analyzed the histochemical localization for every individuals obtained from each UFGT promoter-GUS construct. In onions tissue, the expression levels of the P1promoter (-1487/-857) and the P3 promoter (-477/-18) deletions were very low (Figure 5) and CK no histochemical localization was detected. These results suggest that the region–910 to–344 of the UFGT promoter, in which reside HSE, ABRE, MRE and TCA-element (Table 1) responsible for the strong heat, light, and ABA expression in the transgenic onions. The UFGT promoter construct, P2 produced significant GUS activity in response to bending in onions (Figure 5), whereas other constructs did not show high activity. This indicates that the UFGT promoter region between -910 and -344 bp also contains possible bending stress responsive elements in litchi.

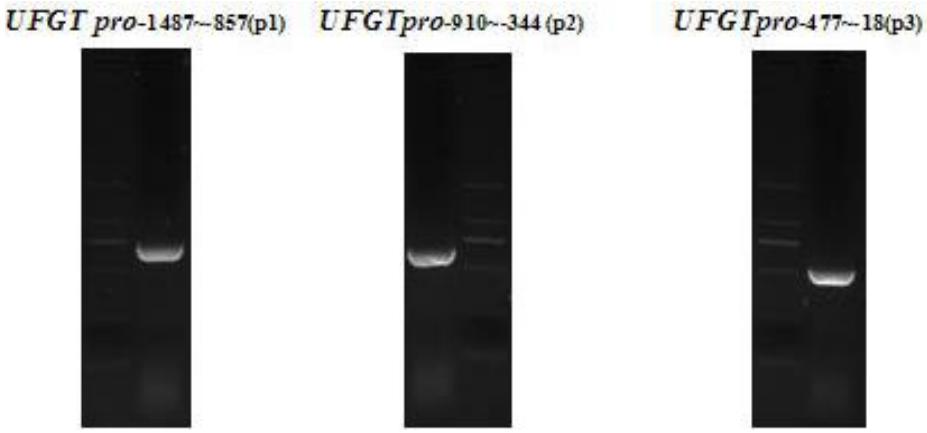

Figure 2. Different fragments of *UFGT* promoter were separated on an agarose gel.

Site Name	Sequence	Function
AE-box	AGAAACAG	part of a module for light response
ATCT-motif	AATCTAATCT	part of a conserved DNA module involved in light responsiveness
Box-4	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	TTTCAAA	light responsive element
CATT motif	GCATTC	part of a light responsive element
ELI-box3	AAACCAATT	elicitor-responsive element
ERE	ATTTCAAA	ethylene-responsive element
GA-motif	ATAGATAA	part of a light responsive element
GATA-motif	AAGATAAGATT	part of a light responsive element
GT1-motif	GGTTAA	light responsive element
I-box	GATAAGATT	part of light responsive element
LTR	CCGAAA	cis-acting element involved in low-temperature responsiveness
Sp1	GGGCGG	light responsive element
TCA-element	GAGAAGAATA	cis-acting element involved in salicylic acid responsiveness
TCCC-motif	TCTCCCT	part of a light responsive element
TCT-motif	TCTTAC	part of a light responsive element
Chs-CMA1a	TTACTTAA	part of a light responsive element
CCAAT-box	CAACGG	MYBHv1 binding site
HSE	CAAAAATTT	cis-acting element involved in heat stress responsiveness
WUN-motif	AAATTTCCC	wound-responsive element
ABRE	GCCACGTTGGA	cis-acting element involved in the abscisic acid responsiveness
MRE	AACCTCA	MYB binding site involved in light responsiveness
TGACG-motif	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness

Figure 3. Regulatory motifs found in the *UFGT* promoter region.

In this study, we isolated the promoter of UFGT from litchi. We isolated the four-franking regions of UFGT from the genomic library of litchi by TAIL-PCR, based on the sequences for the coding regions of UFGT gene. The

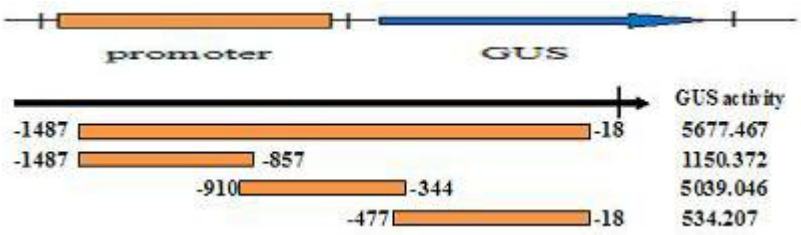

Figure 4. GUS activity of different fragments of litchi gene promoter.

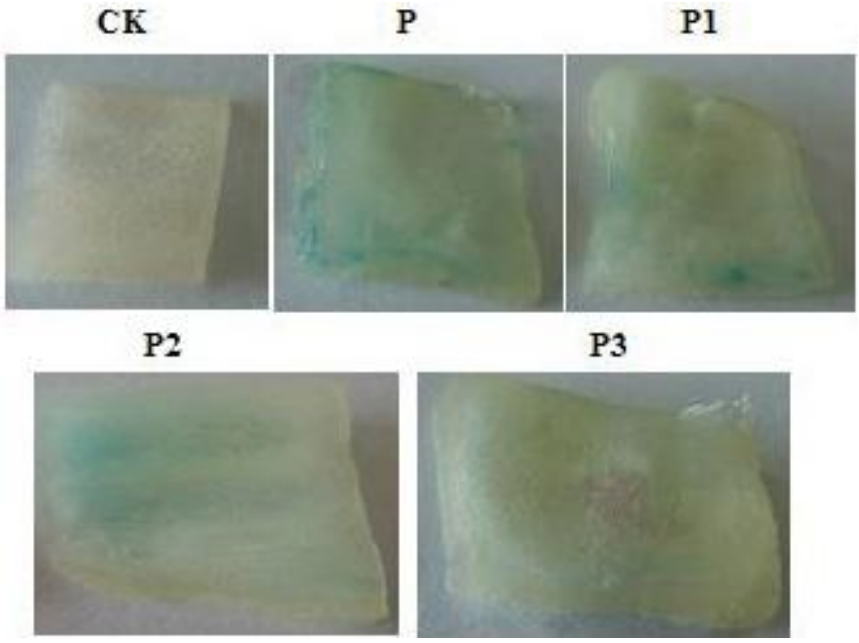

Figure 5. Histochemical analysis of GUS activity of litchi *UFGT* promoter in onions (*Allium cepa* Linn.). No promoter was fused to a GUS reporter gene for CK.

functional motifs of the UFGT gene promoter was predicted by PlantCARE. Promoter deletion analysis of UFGT showed that P2 (-910/-344) promoter-driven GUS activity was detected in response to ABA stress in onions. The expression of UFGT is strongly associated with the anthocyanin accumulation in “Feizixiao” litchi by ABA treatment. The expression of UFGT and anthocyanin were affected by bagging in “Feizixiao”litchi. The induction of UFGT-910 activity under bending stress suggested that the activation of promoter under bending stress might be one of important regulatory mechanisms to response to the stress in onions. The UFGT promoter –910 to –344 contained ABRE, MRE and TCA-element, also have multiple functions in controlling the expression of UFGT under various conditions in litchi. The results suggested that the region of -910 and -344 in the UFGT promoter contains a putative positive regulatory element in the expression of UFGT gene. R2R3-MYB family has important roles in the expression of genes encoding the enzymes in the anthocyanin metabolism via interaction of AC elements (Stracke et al., 2001). Previous study showed that the ectopic expression of PtMYB4 in transgenic tobacco enhanced the transcription of several genes for lignin biosynthetic enzymes (Patzlaff et al., 2003). Recently, several MYBs expressed in differentiating xylem has been isolated from Picea glauca; PgMYB1, PgMYB2, PgMYB3, PgMYB4 and PgMYB8 from *Picea glauca* (Moench) Voss (Bedon et al., 2007). Further studies will be needed to identify conclusively that the transcription factors control the UFGT gene transcription in litchi. In conclusion, our study showed that UFGT promoter from litchi directs the GUS reporter gene expression in onion. Deletion analysis revealed that the promoter region between -910 and -344 was necessary for its function. This study provides a candidate of cis-regulatory elements that control anthocyanin biosynthsis gene expression from litchi and would contribute to the molecular mechanism of litchi coloring.

Conflict of Interest

The authors have not declared any conflict of interest.

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Differential response of early and intermediate flowering strawberry cultivars to nursery late-season nitrogen applications and digging date

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The response of 'Ventana', an early flowering cultivar, and 'Camarosa', an intermediate flowering cultivar, to nursery late-season nitrogen (N) applications and digging date were studied in strawberry (*Fragaria* x *ananassa* Duch). Two experiments were conducted. In the first experiment, runner plants dug on September 20 and October 11 from a high-latitude nursery in California, were established in growth chambers set at 25°/15ºC day/night temperature, 12-h photoperiod, and grown for 90 days. Compared to the first experiment, in the second experiment plants received extra N (foliar-applied) in the nursery in late summer, and runner plants were not grown in GC but in open field (Irvine, California). In the second experiment, runner plants were dug on Sept 20 and Oct 2. In both experiments, plants dug in September were exposed to ~100 chilling units (CU: hours ≤7.2°C) and plants dug in October were exposed to ~300 CU. As a result, October-dug plants had greater crown and root dry weight, and greater concentration of starch and total nonstructural carbohydrates (TNC) in leaves, crowns and roots, compared to September-dug plants. In control plants, from September to October, root TNC concentration increased in 'Camarosa' from ~6% to ~11%, and in 'Ventana' from ~14% to ~21%, and leaf N concentration ranged from 1.47 to 1.81% in 'Camarosa', and from 1.60 to 1.96% in 'Ventana'. Late-summer N applications increased plant N concentration and early-season yields. Late-summer nursery N applications reduced dead leaf biomass (DLB) and dead leaf area (DLA) in both cultivars, although 'Ventana' had lower DLB and DLA than 'Camarosa'. 'Ventana' had a greater leaf number and flowered earlier, and had greater early fruit production than 'Camarosa'. The genetic earliness of 'Ventana' would be correlated with the potential of the plant for accumulation of higher initial levels of leaf N and root TNC, and for having greater leaf longevity, compared to 'Camarosa'.

Key words: *Fragaria* x *ananassa* Duch., foliar urea, carbohydrates, chilling, plant maturity, early flowering cultivars.

INTRODUCTION

Successful plant establishment, early growth and early fruit production in strawberry (*Fragaria* x *ananassa* Duch.) have been related to total nonstructural carbohydrate (TNC) and nitrogen (N) reserves (Bringhurst et al., 1960;

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Larson, 1994; Kirschbaum et al., 2010a). Plants with high root TNC concentration rapidly generate feeder roots (Schupp and Hennion, 1997), providing resources for flower bud initiation (Long, 1935) and early fruit development (Nishizawa and Shishido, 1998).

In the northern hemisphere, N and TNC storage process in roots and crowns of strawberry plants starts in September with the onset of chilling temperatures, and these nutrients are remobilized for bud and new leaf development in spring, as reviewed by Kirschbaum et al. (2010b).

In previous experiments conducted in California, strawberry runner plants cv. 'Camarosa' dug from HL nurseries in early October had enhanced vigor and greater early season fruit production than runner plants dug 2-3 weeks earlier in September, as a result of longer exposures to nursery chilling and decreasing photoperiods (Kirschbaum, 2012). Thus, early season fruit production in 'Camarosa' was also successfully increased as a result of late-summer nursery foliar-N applications (Kirschbaum et al., 2010a). Nevertheless, it is uncertain if both environmental factors will induce the same response in other strawberry cultivars since in this species the genotype x environment interaction is strong. For instance, one study showed that in order to accumulate ~40 $mg.g^{-1}$ FW of starch, in Spain, 'Camarosa' needed 400 chilling hours or units (CU) while 'Pajaro' required 700 CU (Lopez et al., 2002). 'Ventana', a cultivar released by the University of California, is characterized by greater early season fruit production compared to 'Camarosa' (Shaw and Larson, 2002), becoming an feasible cultivar for studying the role of nursery chilling and TNC and N reserves in early season fruit production.

In California, high-latitude strawberry nursery managers usually terminate N applications by the beginning of August in an effort to reduce vegetative growth and "harden off" developing runner plants. This hardening off process was thought to increase TNC accumulation, and reduce susceptibility to disease and transplant stress (Kirschbaum et al., 2010b); however, in a previous study with 'Camarosa', which is intermediate cultivar, we found out that late season nursery N applications were beneficial for early fruit production (Kirschbaum et al., 2010a). In light of these findings, we would like to assess if this is also valid for other cultivars with a different production cycle such as 'Ventana', which is an early cultivar.

The objectives of this work were 1) to study the comparative growth and developmental responses of 'Ventana' and 'Camarosa' to progressive exposure to decreasing temperatures and photoperiods, 2) to compare the responses of the two cultivars to late summer N fertilizer applications and digging date in terms of tissue N and TNC partitioning, and 3) to relate the effects of initial tissue TNC and N contents of nursery plants to subsequent fruit production patterns in the fruiting field.

MATERIALS AND METHODS

Nursery experiments

Nursery treatments were applied in a commercial runner plant propagation nurseries near Dorris (41°58'N, 121°55'W, 1292 m elevation), California. Cold-stored mother plants were used as nursery stock. In 2003, 'Camarosa' plants were established on 45-cm-in-row plant spacing and 'Ventana' on a 40-cm-in-row plant spacing on Apr 4. In 2004, 'Camarosa' and 'Ventana' planting dates were Apr 14 and 18, respectively. Water was supplied by drip irrigation. Plants were grown with standard commercial nursery management, including application of 224 kg N/ha, applied preplant (March) and through the drip irrigation system (May through the first week of August). In the 2004 experiments, N was applied foliarly in late summer. The N formulation was UAN 32 (1% concentration), and it was applied on Sep 6, Sep 13 and Sep 20, 2004 at rates of ~27 kg N/ha each. Second-daughter plants (D2, the 2nd runner plants that develop in a series from stolons of the mother plant) were randomly harvested on Sep 20 and Oct 11, 2003, while first-daughter (D1) and D2 plants were harvested on Sept 20 and Oct 2, 2004. In 2004, plants dug on Sep-20 received two applications of foliar N of 27 kg N/ha each, and plants dug on Oct-2 received three applications of 27 kg N/ha each. Ten plants of each daughter order and nursery N treatment were cold stored at 1°C for 3-4 days, washed thoroughly, dissected into leaflets, petioles, crown and root tissues, and dried at 65°C for 4 days. For all plants, crown diameter was determined prior to drying. In 2004, after drying and dry mass (DM) determination, plants were analyzed for soluble and non-soluble total nonstructural carbohydrates, and total N. The analytical procedure for TNC consisted of enzymatic starch hydrolysis with amyloglucosidase, followed by high performance liquid chromatography (HPLC) for analysis of sugars (Smith, 1969). Total N analysis was performed with a Carlo Erba (Italy) elemental analyzer.

Growth chamber experiments

Bare-root 'Camarosa' and 'Ventana' D2 plants, consisting of leaves, crowns and roots were harvested from a HL nursery near Dorris, California on Sept 20 and Oct 11, 2003, and leaves were removed from all the plants after digging. Plants were planted in rectangular pots 13x13x15 cm (long:wide:deep) filled with "Sunshine mix" (Sun Gro Horticulture Inc., Canada) potting media, which consisted of sphagnum peat moss, perlite, dolomite limestone and gypsum. A teaspoon of controlled-release fertilizer (22-7-10 "Agriform", Grace-Sierra Horticultural Products Co., USA) was applied to each pot at time of planting. Plants were placed in controlled environment chambers, where temperature and photoperiod were set at 25/15ºC day/night and 12 h, respectively. Plants were watered every 2-3 days. Flower emergence date and weight of harvested ripe fruit were progressively recorded for 90 days, when plants were removed from the growth chambers, dissected into component tissues (reproductive, petiols, leaflets, crown, root and fruit) and oven-dried at 65°C for 4 days. Crown diameter, leaf area, and the number of leaves, flowers and fruits (per plant) were measured prior to drying.

Fruiting field experiments

Bare-root D1 and D2 'Camarosa' and 'Ventana' plants consisting of leaves, crowns and roots were harvested from the nursery on Sept 20 and Oct 2, 2004, cold stored at 1°C for 3-4 days and planted in experimental plots at the University of California's South Coast Research and Extension Center, Irvine (33°39'N, 117°41'W). Preplant soil fumigation was applied using a (wt:wt) mixture of 2

Table 1. Cumulative chilling units (CU) corresponding to each plant sampling date in Dorris (California), 2003 and 2004.

Sampling date	Cumulative chilling units (hours at T≤7.2°C)
2003	
20 Sept	116
11 Oct	308
2004	
17 Sept	132
01 Oct	295

methyl bromide : 1 chloropicrin at a rate of 392 kg.ha^{-1}. Plants were established in offset 4-row beds 162 cm wide x 40 cm high using a 40-cm in-row plant spacing (61,250 plants/ha). Each plot consisted of 10 plants of a unique daughter order (D1 and D2), digging date and N treatment. The experimental setup was a completely randomized design with 3 replications. All plots were maintained according to recommendations for California commercial winter plantings (Welch, 1989). Fruit were harvested weekly from Dec 21, 2004 to Apr 11, 2005, using commercial fruit maturity standards. Fruit yields were determined for each plot on a per-plant basis. Canopy diameter as well as leaf and flower numbers were determined prior to fruiting on 11 Nov 2004. Data were subjected to analysis of variance and means were separated using SAS (SAS Institute, 2003).

RESULTS

Accumulated CU in Dorris were very similar in the 2003 and 2004 nursery seasons (Table 1).

First experiment series

Digging date had a significant effect on leaflet, crown and total plant DM (Table 2). In general, a delayed nursery digging date resulted in greater leaf, petiole, crown, root and total plant DM of 'Ventana' for both D1 and D2 plants. In 'Camarosa' the response was similar to 'Ventana' except later-dug D1 plants, which had less leaflet and petiole DM than early-dug D1 plants resulting in decreased plant DM. 'Ventana' plants had a significantly larger root biomass and crown diameter than 'Camarosa' plants. Daughter plant order affected plant DM, crown diameter, leaf number and leaf area (LA) (Tables 2 and 3). First daughter plants were larger than D2 plants in both cultivars. Digging date did not significantly affect crown diameter, number of leaves or leaf area (LA).

In growth chamber experiments, a delay in nursery digging (Table 4) resulted in reduced time to flowering. Cultivar also influenced the time from planting to flowering with 'Ventana' flowering earlier than 'Camarosa' (Table 4). After a 90-day growth period, 'Camarosa' and 'Ventana' plants dug in October had greater flower number, fruit number, fruit fresh weight, fruit DM and reproductive DM compared to plants dug in September (Table 5). Reproductive DM included the whole fruit cluster (pedicel, peduncles, and flowers and fruits at different developmental stages). In general, vegetative traits such as crown diameter, crown DM, leaf number, petiole DM, leaflet DM, LA and root DM were not significantly influenced by digging date. The root:shoot DM ratio was reduced in the plants from the later digging date. After a 90-day growth period, plants dug in October had larger biomass than September-dug plants. Leaf number, fresh fruit weight and fruit DM were significantly greater ($p=0.01$, $p=0.01$ and $p=0.05$, respectively) in 'Ventana' than in 'Camarosa' (data not shown).

Second experiment series

A delay in digging date resulted in significantly increased crown and root biomass for both cultivars and for both daughter plant orders (Table 6). In general, 'Ventana' had greater crown DM than 'Camarosa', but 'Camarosa' had greater root DM.

Late-summer foliar N applications did not affect leaf, crown, root, or plant biomass, crown diameter and LA, but resulted in increased leaf number (Table 7) and decreased dead leaf DM, dead LA (DLA) and DLA:LA ratio at the time of nursery digging (Table 8). First daughter plants had greater dead leaf DM and DLA than D2 plants. 'Camarosa' had greater dead leaf DM, DLA and DLA:LA ratio than 'Ventana'. In control plants, dead leaf DM, DLA and DLA:LA ratio were greater in October than in September; however, N-treated plants generally did not increase dead leaf DM, DLA and DLA:LA ratio from September to October.

In general, a delay in nursery digging date resulted in significantly greater crown diameter and leaf number, but had no effect on LA (Table 7). 'Ventana' had greater crown diameter ($p=0.0337$), leaf number ($p=0.0112$) and LA ($p=0.0812$) than 'Camarosa'. There was a significant effect of daughter order on crown diameter with D1 plants having larger crowns than D2 plants.

Table 2. Effects of digging date on dry mass distribution in daughter 1 (D1) and daughter 2 (D2) strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California), 2003.

Cultivar	Daughter	Plant dry mass (g)		Leaflet dry mass (g)		Petiole dry Mass (g)		Crown dry mass (g)		Root dry mass (g)	
		20 Sep	11 Oct	20 Sep	11 Oct	20 Sep	11 Oct	20 Sep	11 Oct	20 Sep	11 Oct
Camarosa	D1	7.35[1]	6.61	3.93	3.34	1.84	1.61	0.41	0.46	1.17	1.20
	D2	2.65	3.16	1.25	1.50	0.60	0.78	0.18	0.21	0.63	0.46
Ventana	D1	4.74	9.24	2.20	4.71	1.11	1.86	0.23	0.60	1.19	2.08
	D2	3.94	5.02	2.12	2.67	0.88	1.07	0.14	0.32	0.80	0.96
						Pr> F					
Digging date (Dd)		*		**		*ns*		**		*ns*	
Cultivar(C)		*ns*		*ns*		*ns*		*ns*		*	
Daughter(D)		**		**		**		**		**	
DdxC		*		*		*ns*		**		*ns*	
DdxD		*ns*		*ns*		*ns*		*ns*		*ns*	
CxD		*ns*		*ns*		*ns*		*ns*		*ns*	
DdxCxD		*ns*		*		*ns*		*ns*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

Compared with controls, late-season N applications significantly enhanced N concentrations in leaves (Table 9), crowns (Table 10) and roots (Table 11), and, in general, decreased starch and TNC concentrations in leaves, crowns and roots. Crown tissue glucose concentration decreased as a result of N applications in seven out of eight mean comparisons; however, glucose in other tissues, as well as fructose and sucrose in general, did not show a defined trend in response to N applications.

Digging date had a significant effect on N concentration in leaves (Tables 9), generally decreasing from September to October in all tissues. However, for N-treated plants, little or no decrease in N was observed. Thus, the process of reduction of N concentrations observed in crowns and roots of control plants was reversed by late-summer N applications (Tables 10 and 11).

'Ventana' had greater concentrations of root glucose, fructose and sucrose, and greater concentrations of starch and TNC than 'Camarosa' (Table 11), while plants of 'Camarosa' maintained higher concentrations of starch in leaves than plants of 'Ventana' (Table 9).

In fruit evaluation plots, plants that had received late-summer N applications in the nursery had increased canopy diameters and leaf and flower numbers (Table 12), and increased early season yields (to 21 Feb) compared with control plants (Table 13). However, nursery late-summer N applications did not significantly affect total season fruit production. For 'Ventana' plants treated with foliar N in the nursery, early season marketable yields increased by 3 and 41% compared to controls, for plants planted on Sept 24 and Oct 5, respectively. Plants dug in September started to produce fruit on Dec 21, 2004 and had greater early season fruit production in terms of yield and fruit number compared to October plantings, which commenced fruit production on Jan 4, 2005. 'Ventana' outyielded 'Camarosa' in early season fruit production and had greater marketable yield, mean fruit weight, marketable fruit number and better appearance.

Table 3. Effects of nursery digging date on crown diameter, number of leaves and leaf area of daughter 1 (D1) and daughter 2 (D2) strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California), 2003.

Cultivar	Daughter	Crown diameter (mm)		Number of leaves/plant		Leaf area (cm^2)	
		20 Sept	11 Oct	20 Sept	11 Oct	20 Sept	11 Oct
Camarosa	D1	14.8[1]	15.6	7.0	5.9	631	607
	D2	13.3	11.2	4.7	4.2	343	299
Ventana	D1	16.2	17.8	6.1	6.7	712	772
	D2	12.4	13.2	4.3	4.8	333	420
				P>F			
Digging date (Dd)		*ns*		*ns*		*ns*	
Cultivar(C)		*		*ns*		*ns*	
Daughter(D)		**		**		**	
DdxC		*ns*		*		*ns*	
DdxD		*ns*		*ns*		*ns*	
CxD		*ns*		*ns*		*ns*	
DdxCxD		*ns*		*ns*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at p < 0.05, 0.01 and non-significant, respectively.

Table 4. Effects of planting date and cultivar on the time elapsed from planting to flowering of strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California) on Sept 20 and Oct 11, 2003, and established in growth chambers (on Sept 24 and Oct 15, 2003.

Source of variation	Time from planting to flowering (d)	*P>F*
Planting date		
24 Sept	58.34[1]	**
15 Oct	47.20	
Cultivar		
Camarosa	53.69	*
Ventana	50.68	

[1]Analysis of variance: * and **, significant at p < 0.05 and 0.01, respectively.

Table 5. Effects of digging date and cultivar on growth and development of runner plants dug from a HL nursery near Dorris (California) and grown in growth chambers for 90 days (2003).

Variable	Camarosa			Ventana		
	20 Sept		11 Oct	20 Sept		11 Oct
Crown Diameter (mm)	13.2[1]	*ns*	12.8	13.8	*ns*	14.1
LA (cm^2)	466	*ns*	494	568	*ns*	638
Leaves/plant	7.8	*ns*	8.3	10.3	*ns*	12.0
Flowers/plant (FL)	4.2	*ns*	5.7	1.7	*	4.7
Fruits/plant (FR)	3.0	**	5.2	3.7	*ns*	3.3
FL+FR	7.2	*	10.8	5.3	*	8.0
Whole Plant DM (g)	6.3	**	8.5	7.7	**	10.7
Reproductive DM (g)	1.4	**	3.1	1.9	**	4.4
Petiols DM (g)	0.67	*ns*	0.84	0.80	*ns*	0.77
Leaflets DM (g)	2.6	*ns*	2.8	3.0	*ns*	3.4
Crown DM (g)	0.26	**	0.40	0.37	*ns*	0.44

Table 5. Cont.

Root DM (g)	1.5	*ns*	1.4	1.7	*ns*	1.6
Fruit FW (g)	5.6	**	26.8	16.4	**	42.8
Fruit DM (g)	0.6	**	2.4	1.3	**	3.6
Root:Shoot DM ratio	0.30	**	0.18	0.28	**	0.18

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

DISCUSSION

Late-season nursery N applications increased early-season fruit production for strawberry plants dug from a HL nursery and planted in fruit evaluation plots in southern California. These results support the concept that early strawberry fruit production is enhanced by increasing N fertilization near the end of the nursery runner propagation period (Kirschbaum et al., 2010a), but cultivar and digging date can alter the response. In a previous report, October-dug 'Camarosa' plants treated in late-summer with foliar N in the nursery had, on average, 22% greater early season yields than control plants (Kirschbaum et al., 2010a). In the present study, 'Camarosa' treated with late-season foliar N had 12 and 22% greater yield compared to controls for plants dug on Sept 20 and Oct 2, respectively. 'Ventana' plants treated with late-season foliar-applied N had 7% and 36% greater early-season marketable yields compared with controls for plants dug on Sept 20 and Oct 2, respectively.

Fruit number per plant increased with nursery foliar-N treatments and this increase accounted for the greater early yields obtained from N-treated plants as average fruit weight was not affected by nursery N treatment. Whole-season yields were not altered significantly by N treatments. Consistent with previous work (Kirschbaum et al., 2010a), this research indicates that late-summer applied N accelerates plant growth and development resulting in greater early yields without affecting plant yield potential, since whole-season yields were not influenced by N treatments.

Concerns about late season N fertilization in strawberries have been related to the development of excessive foliage, increased susceptibility to pathogens, fruit softening, delayed flowering and ripening, and lower yields (Albregts and Howard, 1987; Daugaard, 2003; Hancock, 1999; May and Pritts, 1990; Miner et al., 1997; Tanaka et al., 2002; Yamasaki et al., 2002). However, in our experiments, we did not observe any of these symptoms and fruit quality attributes such as mean fruit weight, appearance and firmness were not altered significantly by N treatments.

Increased plant vigor due to greater initial runner plant biomass may explain the occurrence of greater early season yields, but we did not observe significant effects of late-season N applications on runner plant biomass or LA. Although N-treated runner plants had less dead leaf DM and leaf area (DLA), and decreased DLA:LA ratios than control plants, it would seem that the contribution to LA of the greater number of senescent leaves due to N application was not significant. The fact that 'Ventana' had significantly less dead leaf DM and DLA as well as a lower DLA:LA ratio than 'Camarosa' may indicate that 'Ventana' leaf longevity is greater than that of 'Camarosa'. Similarly, late-summer N applications might have resulted in increased leaf longevity and a decreased rate of leaf senescence, as has been observed in other species (Erley et al., 2002).

Late-season N applications had a significant effect on plant N and TNC concentration and partitioning, but cultivar and digging date modified these responses. Late-season N applications significantly enhanced N concentration in leaves, crowns and roots, and, in most cases, decreased starch and TNC concentrations in the same organs, which is consistent with previous findings of our research group (Kirschbaum et al., 2010a). Other authors have reported similar results in other temperate fruit crops, proposing that a portion of the carbon from TNC is incorporated into storage amino acids (arginine) and storage proteins, decreasing the carbon stored as starch, glucose and fructose (Bi et al., 2004; Cheng et al., 2004). This may be also the case of strawberries and would explain the decrease in TNC we observed in strawberry plants receiving late-season N.

In the present study, significant effects of cultivar on leaf N concentration were observed. In control plants, leaf N concentration of 'Camarosa' ranged from 1.47 to 1.81% and in 'Ventana' from 1.60 to 1.96 %, showing that nearly all control plants were N-deficient according to the standard sufficiency range for total N in strawberry leaf blades (2.0-2.8 % dry weight; Pritts and Handley, 1998). Late-season N applications resulted in leaf N concentrations in the sufficient range which apparently impacted plant vigor after plants were established in the fruiting field.

The results of this study are also consistent with the hypothesis than TNC accumulation in reserve organs of strawberry plants correlates CU accumulation and decreasing photoperiods (Kirschbaum et al., 2010b). Digging date affected TNC concentration in leaves, crowns and roots, and TNC concentration was greater on Oct 2 than on Sept 20 in all plant tissues. 'Camarosa' root TNC concentration increased from 6.17 to 10.90% in D1

Table 6. Effects of late season N application on dry mass partitioning of daughter 1 (D1) and daughter 2 (D2) strawberry (‘Camarosa’ and ‘Ventana’) plants dug from HL nurseries near Dorris (California), 2004.

Cultivar	Digging date	Daughter	Plant dry mass (g)		Leaflet dry mass (g)		Petiole dry mass (g)		Crown dry mass (g)		Root dry mass (g)	
			N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
Camarosa	20 Sep	D1	9.05	8.47	4.20	4.41	2.97	2.55	0.65	0.54	1.23	0.98
		D2	5.87	4.30	2.91	2.08	1.67	1.30	0.41	0.31	0.88	0.69
	2 Oct	D1	9.35	8.72	4.33	4.00	2.97	2.70	0.78	0.69	1.29	1.32
		D2	6.91	7.42	3.43	3.73	1.73	1.92	0.47	0.53	1.27	1.25
Ventana	20 Sep	D1	7.31	8.07	3.42	4.36	2.41	2.47	0.72	0.52	0.76	0.72
		D2	5.86	6.48	3.18	3.55	1.57	1.70	0.42	0.41	0.69	0.82
	2 Oct	D1	9.33	9.21	4.28	4.68	3.08	2.80	0.79	0.76	1.18	0.99
		D2	8.89	7.68	4.94	3.72	2.33	2.25	0.64	0.64	0.97	1.08
							Pr> F					
Digging date (Dd)			**		*		**		**		*ns*	
Cultivar (C)			*ns*		*ns*		*ns*		*		*	
Daughter(D)			**		**		**		**		*	
Nitrogen (N)			*ns*		*ns*		*ns*		*ns*		*ns*	
DdxC			*		*ns*		*ns*		*ns*		**	
DdxD			*ns*		*ns*		*ns*		*ns*		*	
DdxN			*ns*		*ns*		*ns*		*ns*		*ns*	
CxD			**		*ns*		*ns*		*ns*		*ns*	
CxN			*ns*		*ns*		*ns*		*ns*		*ns*	
DxN			*ns*		*ns*		*ns*		*ns*		*	
DdxCxDxN			*ns*		*ns*		*ns*		*ns*		**	

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

plants, and ‘Ventana’ root TNC concentration increased from 13.67 to 21.33%, for plants dug in September and October, respectively. This is in agreement with previous studies where ‘Camarosa’ TNC concentration increased from ~6 to ~10%, and ‘Selva’ (a day-neutral cultivar) from 4 to 14% (Kirschbaum et al., 2012), for September and October, respectively. According to our experimental results, ‘Ventana’ has the greatest levels of TNC among this group of cultivars and is the earliest.

Currently, recommended planting dates for ‘Ventana’ in southern California are around Oct 1 (Kirk Larson, personal communication). However, prior to Feb 21, 2004, ‘Ventana’ control plants, planted on Sept 24, produced ~50% more yield than Oct-5-planted plants. As reported previously (Baum, 2005), we also observed that early season fruit production and total season fruit production were greater in ‘Ventana’ than in ‘Camarosa’. ‘Ventana’ also overcame ‘Camarosa’ in total season average fruit size, appearance and firmness.

The number of leaves and LA in the fall has been correlated with yield the following spring in biennial strawberry crops (Sproat and Darrow, 1935). We observed that plants receiving nursery

Table 7. Effects of late season N applications on crown diameter, number of leaves and leaf area of D1 and D2 strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California), 2004.

Cultivar	Digging date	Daughter	Crown diameter (mm)		Number of leaves/plant		Leaf area (cm^2)	
			N0	N1	N0	N1	N0	N1
Camarosa	20 Sept	D1	15.4[1]	15.8	4.0	4.8	554	680
		D2	13.8	15.8	3.8	4.6	560	568
	2 Oct	D1	18.1	18.3	4.3	4.4	605	600
		D2	14.7	15.7	3.7	4.9	448	563
Ventana	20 Sept	D1	16.0	15.7	4.2	4.9	581	628
		D2	13.9	15.8	4.0	4.6	589	568
	2 Oct	D1	20.3	18.4	4.8	5.1	735	770
		D2	17.3	17.4	5.3	5.4	661	599
					Pr> F			
Digging date (Dd)				**	*		*ns*	
Cultivar (C)				*	*		*ns*	
Daughter(D)				**	*ns*		*ns*	
Nitrogen (N)				*ns*	**		*ns*	
DdxC				*ns*	*		*ns*	
DdxD				*ns*	*ns*		*ns*	
DdxN				*ns*	*ns*		*ns*	
CxD				*ns*	*ns*		*ns*	
CxN				*ns*	*ns*		*ns*	
DxN				*ns*	*ns*		*ns*	
DdxCxDxN				*ns*	*ns*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

Table 8. Effects of late season N applications on dead leaf dry mass, dead leaf area and DLA:LA DM ratio of D1 and D2 strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California), 2004.

Cultivar	Daughter	Dead leaf DM (g)		Dead LA (DLA) (cm^2)		DLA/LA (%)	
		N0	N1	N0	N1	N0	N1
				September			
Camarosa	D1	1.53[1]	0.73	172	82	21.5	9.9
	D2	0.54	0.33	73	45	13.5	9.6
Ventana	D1	0.58	0.14	59	14	9.0	2.6
	D2	0.29	0.26	27	24	4.6	4.3
				Pr> F			
Cultivar (C)			**	**		**	
Daughter(D)			**	*		*ns*	
Nitrogen (N)			**	**		*	
CxD			*	*ns*		*ns*	
CxN			*ns*	*ns*		*ns*	
DxN			*ns*	*ns*		*ns*	
CxDxN			*ns*	*ns*		*ns*	

Table 8. Cont.

				October			
Camarosa	D1	1.62	0.73	195	78	23.6	11.1
	D2	0.90	0.45	136	59	23.2	9.4
Ventana	D1	1.07	0.21	110	23	13.8	2.8
	D2	0.39	0.15	48	18	6.2	2.8

	Pr> F		
Cultivar (C)	**	**	**
Daughter(D)	**	*	*ns*
Nitrogen (N)	**	**	**
CxD	*ns*	*ns*	*ns*
CxN	*ns*	*ns*	*ns*
DxN	*	*ns*	*ns*
CxDxN	*ns*	*ns*	*ns*

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

N had a greater leaf number at the time of digging, resulting in larger canopy diameter and greater flower numbers after planting. By Nov 11, September-dug plants also had greater canopy diameter and leaf number than October-dug plants. Similarly, by 11 Nov, 'Ventana' had greater root DM, crown diameter, leaf number and LA at the time of digging than 'Camarosa', and also greater leaf number after planting. These correlations may partially explain the high early- season yields obtained by N-treated, September-dug, 'Ventana' plants.

In contrast to field experiments, growth chamber experiments allowed the evaluation of plant vigor and early season yield potential under environmental conditions that did not change from one digging date to another. Nursery runner plants harvested from a HL nursery in 2003 accumulated ~100 h of chilling when dug on Sept 20 and ~300 h when dug on Oct 11. The observed reduction in time to flowering for October-dug plants compared to September-dug plants could be due to an extended exposure to chilling temperatures, shorter photoperiods, and larger initial plant biomass. In general, after a 90-day growth period, plants dug on 11 Oct had greater fruit and flower numbers, fruit weight (fresh and dry), reproductive dry weight and whole plant dry weight than plants dug on 20 Sept and this was consistent for both cultivars.

Our observations of 'Ventana' plants flowering earlier and having greater fresh fruit weight and fruit DM than those of 'Camarosa' agree with the hypotheses that chilling stimulates the development and emergence of previously-differentiated inflorescences and that chilling sensitivity varies among cultivars (Larson, 1994). 'Ventana' seems to have a lower chilling requirement than 'Camarosa'.

Since the California winter strawberry production system relies on fresh-dug runner plants without leaves (Galletta and Bringhurst, 1990), root and crown biomass play a fundamental role in plant establishment and early fruit production. Plants dug at the later dates each season had more crown and root DM than plants dug earlier. Root DM values and accumulation rates followed patterns analogous to previous studies (Kirschbaum et al., 2012; Long and Murneek, 1937; Nishizawa et al., 1998). Increased root DM during Sept-Oct could be attributed to an increased allocation of reserve nutrients to this organ (Bringhurst et al., 1960; Larson, 1994; Long and Murneek, 1937).

Previous work indicated that TNC concentration in strawberry runner plants is positively correlated with exposure to cold temperatures and short photoperiods (Kirschbaum et al., 2010b and 2012; Ruan et al., 2009) and that TNC concentration is negatively correlated with N applications (Kirschbaum et al., 2010a).

Our results support these previous observations and provide evidence for cultivar differences in regard to the effects of chilling on TNC and N accumulation and partitioning, TNC and N dynamics and TNC-N interactions.

Our results support the hypothesis that N reserves are major resources for fall-dug runner plant regrowth after transplanting. Nitrogen reserves have largely been overlooked as having a major role in strawberry transplant establishment and early fruit development in annual production systems (Kirschbaum et al., 2010b). Although details of the N cycling process in strawberries remains to be elucidated, our results indicate that late-season N applications allow plants to remain actively growing during the period of flower differentiation (Hancock, 1999; Long, 1939; Strik and Proctor, 1988), in addition to enhancing mobilization of N to crowns and roots, and with positive effects on early season flowering and fruiting.

Table 9. Effects of late-season N applications on the concentration of N and carbohydrates in leaflets of daughter 1 (D1) and 2 (D2) strawberry plants ('Camarosa' and 'Ventana').

Cultivar	Digging date	Daughter	Nitrogen (% DM)		Glucose (% DM)		Fructose (% DM)		Sucrose (% DM)		Starch (% DM)		TNC (% DM)	
			N0	N1	N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
Camarosa	20 Sep	D1	1.74	2.26	1.7	2.0	1.8	2.2	0.7	0.6	1.3	1.1	5.6	5.6
		D2	1.81	2.46	1.6	1.3	1.7	1.4	1.9	0.6	2.7	1.0	7.9	4.2
	2 Oct	D1	1.68	2.81	1.4	1.8	1.0	1.1	3.2	3.6	2.0	1.1	7.6	7.2
		D2	1.47	1.95	2.1	1.9	1.6	1.3	3.8	3.3	2.4	2.5	10.0	8.3
Ventana	20 Sep	D1	1.65	2.06	2.1	2.7	1.5	2.0	2.1	1.6	1.4	0.7	7.3	6.7
		D2	1.96	2.11	2.5	1.9	1.9	1.3	1.8	3.4	0.8	0.5	6.7	6.6
	2 Oct	D1	1.60	1.94	2.7	1.5	2.0	1.0	3.9	2.6	1.7	1.3	10.7	6.7
		D2	1.65	2.13	2.0	1.8	1.4	1.3	4.7	3.0	1.6	0.8	10.0	6.7
								Pr> F						
Cultivar (C)			*		**		*ns*		*		**		*ns*	
Digging date (Dd)			**		*ns*		**		**		**		**	
Daughter(D)			*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Nitrogen (N)			**		*ns*		*ns*		*ns*		**		**	
CxDd			*ns*		*ns*		*ns*		*		*ns*		*ns*	
CxD			**		*ns*		*ns*		*ns*		**		*ns*	
CxN			**		*ns*		*ns*		*ns*		*ns*		*ns*	
DdxD			**		*ns*		*ns*		*ns*		*ns*		*ns*	
DdxN			**		*ns*		*ns*		*ns*		*ns*		*ns*	
DxN			*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
CxDdxDxN			**		**		*ns*		*ns*		*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

Table 10. Effects of late-season N applications on the concentration of N and carbohydrates in crowns of daughter 1 (D1) and 2 (D2) strawberry plants ('Camarosa' and 'Ventana').

Cultivar	Digging date	Daughter	Nitrogen (% DM)		Glucose (% DM)		Fructose (% DM)		Sucrose (% DM)		Starch (% DM)		TNC (% DM)	
			N0	N1	N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
Camarosa	20 Sep	D1	0.85	1.31	1.6	2.5	1.1	2.0	3.5	1.4	2.8	1.7	9.1	7.2

Table 10. Cont.

Ventana	2 Oct	D2	0.82	0.80	1.7	1.5	1.4	1.0	2.5	3.7	2.9	1.6	8.4	7.7
		D1	0.80	1.80	1.8	1.3	2.0	2.3	3.0	1.4	2.8	2.4	9.6	7.3
		D2	0.69	1.11	2.3	2.0	2.1	2.3	0.8	1.1	4.8	3.4	9.9	8.8
	20 Sep	D1	1.13	1.27	1.9	1.2	1.9	1.4	0.8	0.6	1.8	2.3	6.7	5.7
		D2	1.13	1.14	1.5	1.4	1.7	1.7	1.0	1.1	0.6	1.1	4.3	5.4
	2 Oct	D1	0.93	1.40	1.6	1.1	1.7	1.4	1.7	1.2	2.8	2.4	8.2	6.3
		D2	0.94	1.45	2.0	1.1	2.4	1.3	1.3	1.1	2.8	2.2	9.2	6.0

	Pr> F					
Cultivar (C)	*	**	*ns*	**	**	**
Digging date (Dd)	*ns*	*ns*	**	*	**	**
Daughter(D)	*	*ns*	*ns*	*ns*	*ns*	*ns*
Nitrogen (N)	**	*	*ns*	*	*	**
CxDd	*ns*	*ns*	**	**	*ns*	*ns*
CxD	*	*ns*	*ns*	*ns*	**	*ns*
CxN	*ns*	*	**	*ns*	*	*ns*
DdxD	*ns*	**	*ns*	**	*	*ns*
DdxN	**	*	*ns*	**	*ns*	*
DxN	*	*ns*	*ns*	**	*ns*	*ns*
CxDdxDxN	*ns*	*ns*	*ns*	**	*ns*	*ns*

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

Table 11. Effects of late-season N applications on the concentration of N and carbohydrates in roots of daughter 1 (D1) and 2 (D2) strawberry plants (‘Camarosa’ and ‘Ventana’).

Cultivar	Digging date	Daughter	Nitrogen (% DM)		Glucose (% DM)		Fructose (% DM)		Sucrose (% DM)		Starch (% DM)		TNC (% DM)	
			N0	N1	N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
Camarosa	**20 Sep**	**D1**	1.06	1.44	1.20	0.90	0.70	0.50	1.60	1.73	2.70	2.33	6.17	5.53
		D2	0.96	1.59	0.90	1.40	0.43	0.77	1.13	1.90	2.37	2.50	4.80	6.53
	2 Oct	**D1**	0.86	2.02	1.73	1.03	0.90	0.70	3.10	2.10	3.17	4.63	8.87	8.47
		D2	0.75	1.01	1.00	0.87	0.50	0.37	2.20	1.77	7.27	3.77	10.90	6.83
Ventana	**20 Sep**	**D1**	1.00	1.24	1.87	1.53	0.90	0.80	3.03	2.77	7.07	5.30	13.67	10.67
		D2	1.00	1.04	1.73	1.87	0.73	0.73	3.23	4.10	2.67	4.77	8.33	12.00
	2 Oct	**D1**	0.90	1.31	1.73	1.37	0.97	1.00	3.27	3.33	7.60	5.33	14.33	11.67
		D2	0.77	1.33	1.90	1.87	0.97	1.10	6.10	3.83	10.97	4.73	21.33	12.17

Table 11. Cont.

				Pr> F		
Cultivar (C)	**	**	**	**	**	**
Digging date (Dd)	*ns*	*ns*	**	**	**	**
Daughter(D)	**	*ns*	*	*ns*	*ns*	*ns*
Nitrogen (N)	**	*ns*	*ns*	*ns*	**	**
CxDd	*ns*	*ns*	*	*ns*	*ns*	*ns*
CxD	*	*	*ns*	**	*ns*	*ns*
CxN	**	*ns*	*ns*	*ns*	*ns*	*ns*
DdxD	**	*ns*	*ns*	*ns*	**	**
DdxN	**	*ns*	*ns*	**	**	**
DxN	*ns*	**	*	*ns*	*ns*	*ns*
CxDdxDxN	**	*ns*	**	*ns*	**	**

[1]Analysis of variance: *, ** and ns, significant at p < 0.05, 0.01 and non-significant, respectively.

Table 12. Effects of late season N applications on canopy diameter, number of leaves and number of flowers (to Nov 11, 2004) of strawberry ('Camarosa' and 'Ventana') plants dug from HL nurseries near Dorris (California) and planted in Irvine (California). Daughter plants 1 and 2 were combined for the analysis of variance.

Cultivar	**Digging date**	**Canopy diameter (cm)**		**Number of leaves/plant**		**Number of flowers/plant**	
		N0	**N1**	**N0**	**N1**	**N0**	**N1**
Camarosa	**20 Sept**	24.55[1]	25.43	5.42	6.90	3.30	3.61
	2 Oct	18.54	18.99	4.06	4.41	1.46	2.33
Ventana	**20 Sept**	23.70	26.19	5.88	6.35	2.81	3.67
	2 Oct	17.41	19.55	4.76	5.00	1.41	2.58
				Pr>F			
Nitrogen (N)		**		**		**	
Digging date(Dd)		**		**		**	
Cultivar (C)		*ns*		**		*ns*	
NxDd		*ns*		**		*ns*	
NxC		*		*		*ns*	
DdxC		*ns*		**		*ns*	
NxDdxC		*ns*		*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at p < 0.05, 0.01 and non-significant, respectively.

Table 13. Effects of nursery late-season N applications on early-season and whole-season yields of strawberry runner plants dug from HL nurseries near Dorris (California), 2004. Fruit production plots were established in Irvine (California). D1 and D2 were combined for ANOVA.

Cultivar	Nitrogen	Total yield (g/plant)		Marketable yield (g/plant)		Average fruit weight (g)		Marketable fruits/plant		Appearance		Firmness	
		20 Sep	2 Oct	20 Sep	2 Oct	20 Sep	2 Oct	20 Sep	2 Oct	20 Sep	2 Oct	20 Sep	2 Oct
Early season (Dec-Feb 21)													
Camarosa	**N0**	203	142	126	98	33.1	32.2	3.8	3.1	2.8	3.1	4.0	3.8
	N1	228	173	144	109	32.0	32.0	6.1	3.9	2.8	2.9	3.9	3.8
Ventana	**N0**	267	201	200	138	33.5	35.7	4.5	3.4	3.8	3.6	4.0	3.9
	N1	287	275	206	195	34.1	34.0	6.0	5.8	3.8	3.9	3.9	3.9
							Pr> F						
Nitrogen (N)		**		**		ns		**		ns		ns	
Digging date(D)		**		**		ns		**		ns		ns	
Cultivar (C)		**		**		ns		**		**		ns	
NxD		ns		ns		ns		ns		ns		ns	
NxC		ns		ns		ns		ns		*		ns	
DxC		ns		ns		ns		ns		ns		ns	
NxDxC		ns		*		ns		*		*		ns	
Total season (Dec-Apr 11)													
Camarosa	**N0**	1023	848	677	586	29.9	29.6	22.7	19.9	2.6	2.7	3.7	3.7
	N1	1044	904	673	615	28.7	29.8	23.4	20.7	2.6	2.8	3.6	3.7
Ventana	**N0**	1049	833	778	608	30.8	32.2	25.3	18.9	3.8	3.7	3.8	3.8
	N1	1015	889	736	667	30.8	30.9	24.0	21.6	3.8	3.9	3.8	3.8
							Pr> F						
Nitrogen (N)		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
Digging date(D)		**		**		*ns*		**		*ns*		*ns*	
Cultivar (C)		*ns*		**		**		*ns*		**		**	
NxD		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
NxC		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
DxC		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	
NxDxC		*ns*		*ns*		*ns*		*ns*		*ns*		*ns*	

[1]Analysis of variance: *, ** and ns, significant at $p < 0.05$, 0.01 and non-significant, respectively.

Conflict of Interest

The authors have not declared any conflict of interest.

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Effect of sunflower stover and nutrients management on soil biological properties and available nitrogen and phosphorus at different stage of pigeonpea growth under pigeonpea-sunflower cropping system

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A fixed plot field experiment was carried out during 2008-09 and 2009-10 at New Delhi, India to study the "effects of sunflower stover, nitrogen and phosphorus management on soil health under pigeonpea-sunflower cropping system''. *Kharif* season experiment in the first year was laid out in split-plot design, assigning sunflower stover incorporation (8 t/ha) and no stover incorporation (control) to main plots and combination of P levels and bio-fertilizers {control, 15 kg P/ha, 15 kg P/ha + phosphate solubilizing bacteria (PSB), and 30 kg P/ha} to sub-plots. The spring season experiment of both years was laid out in split-split plot design in which NP doses to sunflower crop {control, 50% recommended dose (RD) of NP, and recommended dose (RD) of NP (80 kg N + 15 kg P/ha} were applied in sub-sub plots. *Kharif* season experiment in the second year was laid out in split-split plot design to investigate the residual effect of NP doses applied to spring season crop in sub-sub plots. Treatments were replicated thrice during both years. Results reveal that the sunflower stover incorporation resulted in better soil biological properties in terms of dehydrogenase activity, alkaline phosphatase activity and microbial biomass carbon (MBC). Available soil NH_4-N and NO_3-N, available P initially reduced due to sunflower stover incorporation but at latter stages, these nutrients in soil increased. Among the various P levels, application of 30 kg P/ha recorded maximum values of soil dehydrogenase, MBC and available P, NH_4-N and lowest values of alkaline phosphatase and NO_3-N. With respect to the residual effect of nitrogen and phosphorus applied to sunflower, among the various levels of N and P, recommended dose (RD) of N and P resulted in better soil biological properties under study and higher values of available N and P.

Key words: Available nutrients, biological properties, cropping system, nutrients management, sunflower stover management.

INTRODUCTION

Nutrient's mining has occurred in many soils due to lack of affordable fertilizer sources and where meager or no organic residue is returned to the soils. The degradation of soil fertility owing to over mining of nutrients and inadequate replenishment through fertilizers can only be curbed through adoption of integrated nutrient management

(INM) technology. The low-input sustainable agriculture (Grubinger, 1992) and reduced chemical input (Kirchner et al., 1993) concepts focus on the re-consideration of agricultural practices, such as burying crop residues and green manuring, in order to maintain the soil organic matter at adequate levels and preserve the whole soil organic status. Therefore, there is urgent need to use all available resources of nutrient to maintain the productivity and fertility at a required level. Among the available organic sources of plant nutrients, crop residue is one of the most important sources for supplying nutrients to the crops.

Sunflower has potential to yield 4-6 t/ha crop residue. This great amount of crop residues are neither used as feed for livestock nor suitable for fuel due to low energy value per unit mass. However, its residue contain major plant nutrients in the range of 0.45 to 0.60% N, 0.15 to 0.22% P and 1.80 to 1.94% K along with secondary and micronutrients (Babu et al., 2014), so its recycling in the soil may be one of the best alternative practices for replenishing the depleted soil fertility. Decomposition of plant residues is the microbially mediated progressive breakdown of organic material into C (biomass or CO_2) and other nutrients (Kumar and Goh, 2000). Crop residues decompose into two distinct phases, an initial rapid phase, in which about 70% of C initially present in the residues is lost as CO_2, followed by a slower phase during which the more resistant fraction is decomposed (Wang et al., 2004). Residue factors include chemical composition, C/N ratio, lignin content and the size of residue particles (Johnson et al., 2007). C/N ratio of residue is a common indicator of residue quality but is not necessarily an accurate predictor of decomposition rate (Handayanto et al., 1994). The incorporation of crop residues into the soil modifies its chemical and biochemical properties, including soil-enzyme activity (Dick et al., 1983), the behaviour of which has often been related to the amount (Speir and Ross, 1983) as well as to the type of organic matter (Perucci et al., 1984). Soil enzymes play a major role in nutrient availability (Martens et al., 1992). In soils, enzymes may be associated with viable cells, dead cells (abiontic enzymes), cell debris and immobilized enzymes in the soil matrix (Burns, 1982).

Dehydrogenase is considered to play an important role in the initial stages of the oxidation of soil organic matter (Ross, 1971) by transferring hydrogen and electrons from substrates to acceptors. Unlike dehydrogenase activity, which can occur only in viable cells, phosphatases have catalytic capabilities both as endoenzymes and as accumulated exo-enzymes in the soil matrix (Kiss et al., 1975). It is very well known that the activity of soil enzymes is higher in the rhizosphere (Tarafdar and Rao, 1990). Pulses have the tap root system and larger rhizospheric area and more enzymatic activity. Among the pulses, pigeonpea is the one of the most important crop, which plays a pivotal role in maintaining the soil fertility. Due to longer root system of pigeonpea, it is often considered as a natural and/or biological plough. Pigeonpea added up to 60 kg of N/ha to the soil and accumulated up to 6 kg of P/ha (Myaka et al., 2006). Phosphorus is one of the most important primary elements from crop production point of view. It exists in nature in a variety of organic and inorganic forms, but primarily in either insoluble or very poorly soluble inorganic forms (Paul and Clark, 1989). Soluble forms of P fertilizers applied to the soil are easily precipitated as insoluble forms. This often leads to an excess application of P fertilizer to crop land. This unmanaged excess may be both an environmental and economic problem. Phosphate solubilizing bacteria solubilise insoluble P by producing various organic acids including oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glyconic, fumaric, adipic and 2-ketogluconic acid (Moghimi and Tate, 1978). Many researchers proved that PSB plays a key role in soil organic P (P_o) transformations (Frossard et al., 1995) through excretion of phosphatase enzymes (Eichler et al., 2004), mineralization of P from organic sources (Gressel and McColl, 1997), and also synthesis and release of P_o (Oberson et al., 2001). This available P is taken up by plants (Banik and Dey, 1981).

In view of the limited information available on the judicious utilization of locally available crop residue and externally inputs especially fertilizers for long term basis and their effect on soil health, the present investigation “focuses on the effects of sunflower stover and nutrients management on soil biological properties and available nitrogen and phosphorus at different stages of pigeonpea growth under pigeonpea-sunflower cropping system in tropical condition of Delhi, India’’.

MATERIALS AND METHODS

Details of experimental field

A fixed plot field experiment was carried out during *kharif* (21.06.2008 and 24.06.2009) and spring seasons (17.02.2009 and 13.02.2010) of 2008-2009 and 2009-2010 for making a comparative assessment of sunflower stover management, P levels and N doses on the soil health under pigeonpea-sunflower cropping system at research farm of Division of Agronomy, Indian Agricultural Research Institute, New Delhi, situated at a latitude of 28°40’ N, longitude of 77°12’ E and altitude of 228.6 m above the mean sea level (Arabian Sea). The soils of experimental field was sandy clay loam belonging to order Inceptisol and having 145.0 kg/ha alkaline permanganate oxidizable N, 17.5 kg/ha available P, 226.0 kg/ha 1 N ammonium acetate exchangeable K and 0.40% organic carbon. The pH of soil was 7.5 (1:2.5 soil and water ratio). Field capacity,

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permanent wilting point and bulk density recorded were 17.0% (w/w), 6.30% (w/w) and 1.46 Mg/m^3, respectively in 0-15 cm soil depth.

Experimental design and treatments

Kharif season experiment in the first year was laid out in split-plot design, assigning sunflower stover incorporation (8 tonnes/ha) on 50% moisture basis for both years and no stover incorporation (control) to main plots and combination of P levels and bio-fertilizers {control**,** 15 kg P/ha, 15 kg P/ha+ phosphate solubilizing bacteria (PSB), 30 kg P/ha} to sub-plots. Spring season experiments of both years was laid out in split-split plot design in which NP doses to sunflower crop (control, 50% RD of NP, RD of NP (80 kg N+15 kg P/ha) were applied in sub-sub plots. *Kharif* season experiment in the second year was laid out in split-split plot design to investigate the residual effect of NP doses applied to sunflower crop in sub-sub plots. The treatments were replicated thrice during both years. The plot size was 17.4 x 15.0 m for main plot, 2.4 x 15.0 m for sub-plot and 2.40 x 4.0 m for sub-sub plots. Firm seed bed of fine tilth was prepared before sowing the crop. After the harvesting of pigeonpea crop (08.12.2008 and 14.12.2009), sunflower was sown on ridges at row distance of 60 cm. The plan of layout for spring crop was made exactly same as previous crop, only sub-plots were divided into sub-sub plots in sunflower to separate the residual and direct effect of treatments. Starter dose of N (25 kg/ha) was given to pigeonpea and phosphorous through diammonium phosphate was supplied as per treatment. Phosphorus was placed 3-5 cm below the seed with the help of metallic tube attached plough. Sunflower stover of the spring crop of sunflower was chopped with the help of chopper and incorporated in the soil as per treatments (8 t/ha) before the sowing of pigeonpea. Pigeonpea seed were inoculated with Microphos as per treatments. In sunflower crop, 50% RD of NP and RD of NP (80 kg N + 15 kg P/ha) respectively was supplied through urea and diammonium phosphate as per treatments. Pigeonpea 'Pusa 992' seed (15 kg/ha) was sown by 'pora' method in rows 60 cm apart. Plant to plant spacing was maintained 15-20 cm apart by adopting gap filling and thinning at appropriate time. For weed control, pre-emergence spray of stomp (pendimethalin) @ 1.0 kg/ha was done in pigeonpea. Beside, herbicide application one hand weeding was done at 30 days after sowing (DAS). Pigeonpea crop was infested with blister beetle and pod borer. In order to control these insects, two spraying of monocrotophos @ 0.04% were given. Pigeonpea was grown as per recommended practices and were harvested in the first fortnight of November in both year of experimentation.

Studies on soil biological properties

Biological properties of soil such as dehydrogenase activity estimated by the method described by Casida et al. (1964), alkaline phosphatase activity estimated by the method described by Tabatabai and Bremner (1969) and microbial biomass carbon estimated by the method described by Vance et al. (1987) and Numan et al. (1998) was used. These properties were analyzed at 30, 60, 120 DAS and at harvest of pigeonpea. Soil samples were taken from crop root (0-15 cm soil depth) by core sampler. The soil samples were air dried and kept in freezer until the analysis of the parameters.

Studies on soil available N and P

The composite soil samples were collected from 0-15 cm soil profile before sowing and 30, 60, 120 DAS and at harvest of pigeonpea. The soil samples were air-dried, ground and passed through 100 mm mesh sieve and were analyzed for NH_4–N and NO_3–N, P and K. The NH_4–N and NO_3–N were estimated by Magnesium Oxide-Devardas alloy method for soil extract (Keeney and Nelson, 1982) and expressed in kg/ha. The available P content in soil was estimated by Olsen et al. (1954) method and expressed in kg/ha.

Statistical analysis

All the data obtained from the experiment during two consecutive years were statistically analyzed using the *F*-test procedure given by Gomez and Gomez (1984). Critical difference (CD) values at *P*=0.05 were used for determining the significance of differences between means.

RESULTS

Effect of sunflower stover incorporation and nutrient management on selected soil biological properties

Dehydrogenase activity

In general, dehydrogenase activity (µ/g soil/day) increased in both years as the crop advanced in age, dehydrogenase activity comparatively higher during 2009 as compared to 2008 and the highest dehydrogenase activities were recorded at harvesting and the lowest at 30 DAS during both seasons (Table 1). Perusal of data indicates that sunflower stover incorporation recorded significantly higher values of dehydrogenase activities at all the growth stages of pigeonpea *viz.,* 30, 60, 120 DAS and at harvest during both years of experimentation over the control except at 30 DAS in 2008. Significantly higher values of dehydrogenase activities were recorded from all the levels of P over the control at all the growth stages of pigeonpea *viz.,* 30, 60, 120 DAS and at harvest during both seasons. Among the P levels, application of 30 kg P/kg recorded significantly higher values of dehydrogenase activities over the rest of the treatments at 30, 60 DAS and at harvest, being at par with 15 kg P/ha+PSB at 60 and 120 DAS during both the seasons and at harvest during 2008. Except 120 DAS stage, maximum value of dehydrogenase activity was observed with 30 kg P/ha. At 120 DAS, maximum value of dehydrogenase activities was found with 15 kg P/ha+PSB, followed by 30 kg P/ha during both the years of field experimentation. NP doses applied to preceding sunflower had significant residual effect on soil dehydrogenase activities of succeeding pigeonpea under pigeonpea-sunflower cropping system. Residual effect of RD of NP registered the highest soil dehydrogenase activities in succeeding pigeonpea at all the growth stages, however it remained statistically on par with the residual effect of 50% RD of NP at all the growth stages except at 30 DAS.

Alkaline phosphatase activity

Alkaline phosphatase activity (µg p-nitrophenol/g/h) was

Table 1. Direct effect of sunflower stover and P management and residual effect of NP on dehydrogenase activity in soil of pigeonpea.

Treatment	Dehydrogenase activity (µg TPF/g soil/day)							
	30 DAS		60 DAS		120 DAS		At harvest	
	2008	2009	2008	2009	2008	2009	2008	2009
Direct effect of SFS management								
Control	0.033	0.033	2.63	3.99	4.63	5.32	6.45	7.77
SFSI @8 t/ha	0.037	0.042	5.01	5.38	6.98	7.92	8.21	9.17
SEm±	0.001	0.001	0.04	0.04	0.10	0.04	0.15	0.08
CD (*P*=0.05)	NS	0.004	0.25	0.22	0.62	0.26	0.91	0.49
Direct effect of P levels								
Control	0.022	0.036	2.55	3.35	3.90	4.66	5.90	6.76
15 kg P/ha	0.029	0.033	3.92	4.80	5.55	6.11	7.35	8.21
15 kg P/ha+PSB	0.037	0.036	4.25	5.17	7.25	7.98	8.00	9.08
30 kg P/ha	0.051	0.045	4.55	5.42	6.50	7.73	8.07	9.83
SEm±	0.002	0.002	0.21	0.11	0.38	0.09	0.41	0.10
CD (*P*=0.05)	0.006	0.006	0.66	0.34	1.18	0.29	1.25	0.31
Residual effect of NP doses applied to sunflower								
Control	-	0.032	-	4.31	-	6.25	-	8.10
50% RD of NP	-	0.035	-	4.75	-	6.69	-	8.54
RD of NP	-	0.045	-	4.99	-	6.92	-	8.77
SEm±	-	0.002	-	0.10	-	0.13	-	0.09
CD (*P*=0.05)	-	0.005	-	0.28	-	0.37	-	0.27

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

higher during the second year of experiment as compared to first year irrespective of treatments (Table 2). Alkaline phosphatase activity increased with the advancement in crop stages up to 120 DAS and there after the activity declined until maturity. Significantly higher values of alkaline phosphatase activity were recorded with sunflower stover incorporation over control during both seasons at all the growth stages *viz*., 30, 60, 120 DAS and at harvest. All the P levels significantly affected the alkaline phosphatase activity in soil at all the stages during both seasons. Significantly lower values of alkaline phosphatase activity were recorded with 30 kg P/ha at all the growth stages of pigeonpea during both the seasons. In contrast, application of 15 kg P/ha+PSB recorded significantly higher values of alkaline phosphatase activity at all the growth stages over the rest of the P levels during both the years. NP doses applied to preceding sunflower significantly reduced the alkaline phosphatase activity of succeeding pigeonpea under pigeonpea-sunflower cropping system. Residual effect of RD of NP (80 kg N+15 kg P/ha) recorded the lowest values of alkaline phosphatase activity at all the stages but it remains statistically on par with the residual effect of 50% RD of NP at all the growth stages. However, maximum values of alkaline phosphatase activity were observed with control.

Microbial biomass carbon (MBC)

Across the seasons, MBC values (mg/kg of soil) were marginally higher during 2009 than 2008 (Table 3). MBC registered marked increase with the advancement in crop growth stages up to harvest. Over the stages, maximum increase was observed at 120 DAS followed by at harvest. Significantly higher value of soil MBC recorded with sunflower stover incorporation over control during both the seasons at all the growth stages *viz.,* 30, 60, 120 DAS and at harvest, except at 30 DAS during 2008. All the P levels significantly increased the MBC content in soil over the control at all the stages during both seasons, except at 30 DAS during 2008 and at 60, 120 DAS and at harvest during 2009 at these stages, control being statistically at par with 15 kg P/ha. In 2008, maximum values of MBC were recorded with 15 kg P/ha+PSB at 30 and 60 DAS. However, in 2009 maximum values of MBC were recorded with 30 kg P/ha at all the stages *viz.,* 30, 60, and at harvest, except at 120 DAS. But both the

Table 2. Direct effect of sunflower stover and P management and residual effect of NP on alkaline phosphatase activity in soil of pigeonpea.

Treatment	Alkaline phosphatase activity (µg p-nitrophenol/g soil/h)							
	30 DAS		60 DAS		120 DAS		At harvest	
	2008	2009	2008	2009	2008	2009	2008	2009
Direct effect of SFS management								
Control	45.6	48.8	56.0	63.9	102.8	104.8	63.8	64.8
SFSI @8 t/ha	54.8	62.6	70.8	72.3	113.3	117.4	75.3	76.6
SEm±	0.77	0.83	0.71	0.36	1.20	0.48	0.61	0.29
CD (*P*=0.05)	4.66	5.07	4.35	2.17	7.32	2.90	3.73	1.76
Direct effect of P levels								
Control	53.0	59.8	67.5	73.3	113.0	118.5	74.0	76.7
15 kg P/ha	48.5	51.7	60.0	64.7	103.5	108.7	66.0	64.3
15 kg P/ha+PSB	56.5	66.5	72.0	79.7	119.5	120.3	80.5	85.7
30 kg P/ha	42.7	44.8	54.0	54.8	96.0	97	57.5	56.2
SEm±	1.11	1.17	1.23	1.00	1.68	0.69	0.99	0.76
CD (*P*=0.05)	3.41	3.60	3.80	3.09	5.19	2.12	3.04	2.33
Residual effect of NP doses applied to sunflower								
Control	-	57.5	-	70.4	-	113.1	-	73.3
50% RD of NP	-	55.5	-	67.9	-	112	-	70.5
RD of NP	-	54.1	-	66.1	-	108.3	-	68.4
SEm±	-	0.73	-	1.07	-	1.12	-	1.26
CD (*P*=0.05)	-	2.09	-	3.08	-	3.23	-	3.63

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

treatments remain statistically at par during both the years. NP doses applied to preceding sunflower had significant residual effect on MBC in soil of succeeding pigeonpea under pigeonpea-sunflower cropping system. MBC in succeeding pigeonpea was higher under the residual effect of RD of NP (80 kg N+15 kg P/ha) over control at all the stages, but remained at par with 50% RD of NP.

Effect of sunflower stover incorporation and nutrient management on soil available nutrients

Available NH_4-N and NO_3-N in soil at different growth stages of pigeonpea

The data on available NH_4-N and NO_3-N in both years (2008 and 2009) are presented in Tables 4 and 5, respectively. Critical appraisal of data reveals that NH_4-N content in soil was higher at earlier stage and decreased over the season due to nitrification process. In contrast, NO_3-N was low in the early stages of growth and gradually buildup towards the end of the season during both year of experimentation. The effect of sunflower stover incorporation was significant at all the growth stages of pigeonpea on NH_4-N and NO_3-N. At 30 and 60 DAS, NH_4-N values were recorded significantly low under sunflower stover incorporation. At 120 DAS and at harvest, the trend was reversed. NO_3-N was markedly lower under sunflower stover incorporation at all the growth stages than no stover incorporation except at harvest during 2008, where statistically similar values were recorded. Different levels of P also had significant influences on both NH_4-N and NO_3-N during both the season. During first year of experimentation (2008), the maximum NH_4-N was recorded with 30 kg P/ha at all the growth stages, which was at par with 15 kg P/ha+PSB and 15 kg P/ha except at harvest stage, where 15 kg P recorded lower values than 30 kg P/ha. In 2009, markedly higher values of NH_4-N were recorded with 30 kg P/ha over the other levels of P, except 120 DAS, where 30 kg P and 15 kg P/ha+PSB was statistically at par.

With regard to NO_3-N, maximum NO_3-N was recorded in control plot followed by 15 kg P/ha. However, lowest values of NO_3-N were recorded with 30 kg P/ha at all the growth stages. Residual effects of NP doses applied to preceding sunflower crop was not found significant on both NH_4-N and NO_3-N in soil of succeeding pigeonpea at all the growth stages.

Table 3. Direct effect of sunflower stover and P management and residual effect of NP on microbial biomass carbon in soil of pigeonpea.

Treatment	Microbial biomass carbon (mg/kg of soil)							
	30 DAS		60 DAS		120 DAS		At harvest	
	2008	2009	2008	2009	2008	2009	2008	2009
Direct effect of SFS management								
Control	116.25	118.75	124.92	128.25	170.58	171.50	210.92	206.75
SFSI @8 t/ha	121.50	129.31	160.25	162.81	194.25	198.06	229.75	237.56
SEm±	1.43	0.75	0.56	0.55	2.37	0.75	0.72	0.51
CD (*P*=0.05)	NS	4.56	3.42	3.32	14.40	4.56	4.36	3.11
Direct effect of P levels								
Control	109.17	119.39	135.00	140.39	175.17	171.39	215.00	215.89
15 kg P/ha	118.50	121.56	137.50	143.56	177.00	183.56	218.50	221.06
15 kg P/ha+PSB	125.33	127.00	149.83	148.00	188.50	193.00	223.33	224.00
30 kg P/ha	122.50	128.17	148.00	150.17	189.00	191.17	224.50	227.67
SEm±	1.78	0.89	1.67	0.82	2.57	0.75	1.87	1.11
CD (*P*=0.05)	5.50	2.75	5.13	2.51	7.91	2.30	5.76	3.43
Residual effect of NP doses applied to sunflower								
Control	-	122.67	-	144.17	-	183.42	-	220.79
50% RD of NP	-	124.00	-	145.50	-	184.75	-	222.13
RD of NP	-	125.42	-	146.92	-	186.17	-	223.54
SEm±	-	0.73	-	0.64	-	0.63	-	0.70
CD (*P*=0.05)	-	2.09	-	1.85	-	1.80	-	2.01

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

Available P in soil at different growth stages of pigeonpea

Data pertaining to available P in soil at different growth stages of pigeonpea are presented in Table 6. Perusal of data reveals that available P in soil was recorded significantly higher due to sunflower stover incorporation except 30 DAS, where control was superior. Various P levels increased the available P in soil over the control. Among the different P levels significantly higher values of available P were found with 30 kg P/ha, but it remained consistently at par with 15 kg P/ha+PSB at all the stages during both the crop seasons except at harvest in 2009. At this stage, it was significantly superior over all the P levels. N and P applied to preceding sunflower crop in pigeonpea-sunflower cropping system had a significant residual effect on available P in the soil for succeeding pigeonpea. Residual effect of both doses of NP, that is, 50% RD of NP and RD of NP (80 kg N+15 kg P/ha) increased the available P in soil as compared to control. Highest values of available P were registered with the residual effect of RD of NP (80 Kg N+15 kg P/ha), at initial stage of crop (30 and 60 DAS); it was significantly superior over the residual effect of 50% RD of NP. However, at the latter stages (60 DAS and at harvest) residual effect of RD of NP remained at par with the residual effect of 50% RD of NP.

DISCUSSION

Soil biological properties

The data on microbial activity in terms of dehydrogenase activity, alkaline phosphatase activity and microbial biomass carbon during crop growth period were recorded at 30, 60, 120 DAS and at harvest and presented in Tables 1, 2 and 3, respectively. These activities provide the information on the microbial growth and development. Dehydrogenase activity was chosen as an index of microbial activity as it refers to a group of mostly endo cellular enzymes, which catalyze oxidation of soil organic matter (Pascual et al., 1998). In the present study, higher values of dehydrogenase activity, alkaline phosphatase activity and microbial biomass carbon (at harvest) were observed with sunflower stover incorporation. The lowest values were observed initially and then gradual increase was recorded over the period of growth of crop. All these results suggest that adoption of an organic amendment could lead to an increase in the long term sustainability of soil fertility by improving levels of soil organic matter,

Table 4. Direct effect of sunflower stover and P management on available NH_4-N and NO_3-N (kg/ha) at various stages of pigeonpea in 2008.

Treatment	30 DAS		60 DAS		120 DAS		At harvest	
	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N
Direct effect of SFS management								
Control	121.5	44.7	127.9	53.7	89.0	63.1	76.4	67.9
SFSI @8 t/ha	111.4	41.2	112.6	45.0	113.8	45.2	82.2	68.8
SEm±	1.2	0.18	0.70	0.8	0.53	0.25	0.71	0.57
CD (*P*=0.05)	7.3	1.1	4.2	4.9	3.2	1.5	4.3	NS
Direct effect of P levels								
Control	110.3	45.8	114.1	51.4	94.7	57.7	71.4	73.1
15 kg P/ha	115.9	43.3	118.9	50.2	101.1	54.3	77.9	69.5
15 kg P/ha+PSB	118.7	41.6	122.3	48.1	103.9	52.6	82.3	66.2
30 kg P/ha	120.9	41.1	124.2	47.6	105.9	51.9	85.5	64.5
SEm±	1.8	1.0	2.1	0.77	1.98	0.8	1.63	1.00
CD (*P*=0.05)	5.5	3.0	6.5	2.4	6.1	2.6	5.0	3.1

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

Table 5. Direct effect of sunflower stover and P management and residual effect of NP on available NH_4- N and NO_3-N (kg/ha) at various stages of pigeonpea in 2009.

Treatment	30 DAS		60 DAS		120 DAS		At harvest	
	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N
Direct effect of SFS management								
Control	121.2	45.5	127.9	54.1	89.8	63.8	76.8	68.8
SFSI @8 t/ha	111.2	42.0	113.2	46.4	114.4	45.2	82.8	69.8
SEm±	0.29	0.17	0.64	0.24	0.45	0.26	0.53	0.13
CD (P=0.05)	1.76	1.06	3.89	1.45	2.71	1.56	3.23	0.79
Direct effect of P levels								
Control	110.8	46.8	114.7	53.1	95.6	58.4	72.1	73.9
15 kg P/ha	116.7	43.9	119.7	50.9	101.7	55.0	77.9	70.8
15 kg P/ha+PSB	118.9	42.6	122.2	48.8	104.6	53.2	82.8	67.1
30 kg P/ha	121.5	41.8	125.0	48.3	106.6	52.8	86.4	65.3
SEm±	0.68	0.24	0.74	0.35	1.04	0.23	1.04	0.67
CD (P=0.05)	2.10	0.75	2.29	1.08	3.22	0.71	3.22	2.07
Residual effect of NP doses applied to sunflower								
Control	116.6	44.4	119.2	50.7	101.4	55.4	79.3	70.0
50% RD of NP	116.8	43.8	120.5	50.4	102.0	54.9	79.6	69.4
RD of NP	117.5	43.1	121.3	49.7	102.9	54.2	80.5	68.3
SEm±	0.39	0.43	0.41	0.44	0.53	0.41	0.52	0.52
CD (P=0.05)	NS	NS	NS	NS	NS	NS	NS	NS

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

available nutrients and soil microbial activity (Chander et al., 1998). The organic amendments may supply an additional source of labile carbon and their nutrients to soil for microbial growth and activity (Boggs et al., 2000). Increased dehydrogenase activity, alkaline phosphatase activity and microbial biomass carbon due to application of rice straw compost in rice soil was also reported by Goyal et al. (2009). P levels up to 30 kg P/ha increased the values of dehydrogenase activity and microbial biomass carbon at all the growth stages. Balanced

Table 6. Direct effect of sunflower stover and P management and residual effect of NP on available soil P at different stages of pigeonpea.

Treatment	Available P (kg/ha)							
	30 DAS		60 DAS		120 DAS		At harvest	
	2008	2009	2008	2009	2008	2009	2008	2009
Direct effect of SFS management								
Control	21.98	22.08	19.98	20.06	17.58	17.74	16.30	16.86
SFSI @8 t/ha	20.09	20.16	22.17	22.27	19.73	19.91	18.60	19.10
SEm±	0.29	0.16	0.25	0.27	0.11	0.10	0.11	0.12
CD (P=0.05)	1.74	0.95	1.51	1.64	0.68	0.62	0.68	0.76
Direct effect of P levels								
Control	17.33	17.43	17.17	17.29	17.10	17.27	17.00	17.17
15 kg P/ha	20.50	20.50	20.55	20.65	18.42	18.47	17.35	17.69
15 kg P/ha+PSB	22.77	22.85	22.92	23.00	19.28	19.49	17.55	18.09
30 kg P/ha	23.55	23.70	23.65	23.72	19.82	20.09	17.90	18.98
SEm±	0.76	0.37	0.76	0.40	0.61	0.33	0.16	0.20
CD (P=0.05)	2.35	1.15	2.33	1.23	1.89	1.00	0.48	0.62
Residual effect of NP applied to sunflower								
Control	-	20.53	-	20.55	-	18.15	-	17.68
50% RD of NP	-	21.04	-	21.07	-	18.64	-	18.02
RD of NP	-	21.80	-	21.88	-	19.69	-	18.25
SEm±	-	0.20	-	0.17	-	0.30	-	0.15
CD (*P*=0.05)	-	0.59	-	0.48	-	0.87	-	0.42

SFSI: Sunflower stover incorporation, RD of NP: 80 kg N+15kg P/ha; SFS: sunflower stover; DAS: days after sowing CD: critical differences.

fertilization enhanced the microbial biomass, dehydrogenase activity and phosphatase activity, which may be due to the higher production of organic carbon and P fertilization in particular (Chua et al., 2007). In contrast, alkaline phosphatase activity decreased with increasing the levels of P. Tadano et al. (1993) stated that P cycle enzyme activities are inversely related to P availability and when P is a limiting nutrient, its demand increases resulting in an increase in phosphatase activity. This phenomenon can be explained by a competitive inhibition of phosphatase by phosphate ions or by a negative-feedback of phosphate ions on PHO genes resulting in a repression of phosphatase synthesis by microorganisms (Oshima et al., 1996). These results are in close conformity with the findings of Moscateli et al. (2005) and Bhadoria et al. (2011). Residual effect of NP applied to preceding sunflower up to RD of NP (80 kg N+15 kg P/ha) increased the soil dehydrogenase activity and soil microbial biomass carbon and reduced the alkaline phosphatase activity of succeeding pigeonpea under pigeonpea-sunflower cropping system. This was due to the favourable soil condition and improved soil organic matter due to left over soil nutrients. Improvement in soil biological properties due to the balanced inorganic fertilizer was also recorded by Chu et al. (2005) and Masto et al. (2006).

Available NH_4-N and NO_3-N in soil at different growth stages of pigeonpea

As the experiment proceeded, NH_4-N decreased and NO_3-N increased proportionately. NH_4-N was observed higher at early stage and declined thereafter, whereas there was an inverse trend in NO_3-N, which was lower at earlier stage and increased at harvest stage. Sunflower stover incorporation resulted in the lower values of both NH_4-N and NO_3-N in initial stage up to 60 DAS, after that availability of both NH_4-N and NO_3-N were increased. This might be due to immobilization of mineral nitrogen at initial stage due to sunflower stover incorporation. At later stages, increase in mineral N content was an indicator of release of N from crop residue. Similar findings were also observed by Corbeels et al. (2000), Kachroo et al. (2006) and Shrinivas et al. (2006). P levels have significant influence on both NH_4-N and NO_3-N during both season. Maximum NH_4-N was recorded with 30 kg P/ha at all the growth stages followed by 15 kg P/ha+PSB. However, maximum NO_3-N was recorded in control plot followed by 15 kg P/ha. These variations may be assigned to higher amount of N fixed on account of profused root development and better nodulation under P fertilized plots (Patel, 1980). On the other hand, NO_3-N was higher under control plot due to less N fixation and more

nitrification process. Residual effect of NP applied to preceding sunflower crop did not cause any significant difference on both NH_4-N and NO_3-N in soil of succeeding pigeonpea at all the growth stages.

Available P in soil at different growth stages of pigeonpea

Maximum values of available P was observed at 60 DAS there after availability of P in soil decreased. Sunflower stover incorporation caused immobilization of mineral P at initial stage of decomposition and reduced the availability of soil P but in latter stages sunflower stover incorporation caused marked improvement in available P. Similar results were recorded by Sarmah and Bordoloi (1994), Saha et al. (1995) and Lal et al. (2000). It is quite likely as organic additives produce organic acids during the decomposition which increased the availability of phosphorus in soil (Debnath et al., 1991). Various P levels have increased the available P in soil over the control.

Among different P levels, application of 30 kg P/ha and 15 kg P/ha+PSB recorded similar values of available P in the soil at all stages during both the crop seasons. This might be due to increase in amount of root exudates and increased microbial activity leading to greater mineralization of applied and inherent P. Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils (Stevenson, 2005). The PSB dissolve the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Goldstein, 1995; Deubel et al., 2000). Residual effect of both doses of NP up to RD of P resulted higher availability of soil P as compared to control.

This study suggested that sunflower stover incorporation in pigeonpea under pigeonpea-sunflower cropping system improves the soil health which may enhance the agricultural sustainability. Similarly, application of 30 kg P/ha in pigeonpea was found to be best performing with respect to improving the soil health in terms of biological and chemical properties. Residual effect of RD of NP given to sunflower resulted in better soil health of pigeonpea.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Filing considerably breaks seed dormancy of *Berchemia discolour* Hemsley

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In this study, the effectiveness of different pre-treatment methods for the breakings of seed dormancy in *Berchemia discolour* Hemsley seeds was assessed. Viable seeds were randomly sampled and subjected to different pre-treatment methods which include filing, soaking in 98% concentrated sulphuric acid, pre-chilling and boiling. For each treatment, ten seeds were used and three replications were done for each treatment. The investigation revealed that filing enhances seed germination and seed dormancy is likely due to the hard seed coat which has to be weakened or broken gently to avoid embryo damage, thus facilitating germination. Although, filing caused seeds to germinate, the mean germination rate of 13.3% that was achieved is too low to make this investigation conclusive. Further studies similar to this are recommended to improve the germination rate to higher than 30%.

Key words: Dormancy, filing, germination, pre-chilling, pre-treatment.

INTRODUCTION

Berchemia discolour Hemsley (bird plum/brown ivory) is a shrub or tree with a dark flacking bark, with height range of 3-20 m. It is widely distributed, but tends to be more abundant at low altitudes, along rivers and on termite mounds. The tree is browsed by game. Its bark and leaves are used medicinally (van Wyk and van Wyk, 1997; Adebooye and Opabode, 2004; McGaw et al., 2007) and the yellow-brown wood is hard, attractive and suitable for furniture. *B. discolour* fruits are edible, sweet tasting and used in beverage making. The fruits can be eaten fresh, sun dried or boiled with sorghum and the fruit pulp has high vitamin C content (Kamumvuri, 2004).

B. discolour plants produce numerous small fruits susceptible to various mechanisms of dispersal. However, very few, if any plants are evident in a locality reflecting a low germination rate relative to the seed population (van Wyk and van Wyk, 1997). The seeds enter dormancy at maturity and are difficult to germinate under natural environmental conditions due to the seed hard coat (van Wyk and van Wyk, 1997). Seeds require

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a specific trigger or change in the surrounding environment to break dormancy and germinate under favourable conditions (Williams et al., 2003; Zoghi et al., 2011; Long et al., 2014).

Commercial standards for exotic trees would be satisfied by a germination rate of above 90% to minimize seed wastage. Indigenous trees have poor germination rates when compared with exotic ones. An improved germination rate of above 50%, if achieved, would be satisfactory and can be conclusive that this method is effective in breaking seed dormancy and can be utilized commercially (Majaju and Zananga, 2006). Application of the pre-treatment method of filing should be improved so as to achieve a better germination rate. Seed coat impermeability is not the only cause for prolonged and low germination for *B. discolour* and it is recommended that the seeds be chopped at the radicle end followed by soaking in cold water and drying in the sun for 24 h as a method to promote germination (Walck et al., 2012).

Majaju and Zananga (2006), working on indigenous tree species in Zimbabwe, germinated a variety of trees and observed varied germination rates. Though pre-treatments used were not highlighted, the following results were found: *B. discolour* 30% germination over 4 weeks, *Uapaca kirkiana* 70% in 3 weeks, *Pterocarpus angolensis* 40% in 4 weeks, *Azanza garckeana* 65% in 4 weeks, *Brachystegia boehmii* 82% in 3 weeks and *Brachystegia spiciformis* 85% in 4 weeks (Majaju and Zananga, 2006)

B. discolour seeds have hard seed coats that confers physical dormancy and scarification is one of the popular methods commonly used to break dormancy on such types of seeds (Tibugari et al., 2013). Scarification is any process of scratching, breaking or mechanically altering the seed coat to make it permeable to water and gases (Evans and Blazich, 2010;, Zoghi et al., 2011; Paul et al., 2003). Hard seeds can be softened by subjecting them to artificial pre-treatment mechanisms, either via scarification or stratification.

B. discolour is of great value, however its utilization commercially is currently limited because of the problems encountered in the species regeneration through seeds. Establishing *B. discolour* plantations would be of great benefit to communities given that the fruits are produced during the cold and dry seasons when there are few other types of fruits and vegetables and therefore can help to provide a healthy diet (Whyman, 1993). Hence, the main aim of this study was to access the effectiveness of different seed pre-treatment methods in breaking seed dormancy for higher regeneration of the species.

MATERIALS AND METHODS

Seed collection

Sun dried fruits of *B. discolour* were collected from the Mavuradonha and Chimhanda areas in Mt Darwin and Rushinda districts, respectively in Zimbabwe. The seeds were extracted from the fruits by removing the exterior fruit pulp and were taken for viability tests. Seeds from the two areas were bulked and treated as collected from a single population before viability tests were performed.

Seed viability testing

The floatation and observation processes were used to test the viability of the seeds. Seeds were observed thoroughly using a hand lens for cracks or other physical damage by insects or other pests. Cracked seeds and those bored by insects were removed from the samples. Seeds were then placed in a container with a 10 cm water column. The column length ensured a clear distinction between the seeds that sunk and those that floated. According to Visser (1994), the seeds that sank are viable. The test may not be entirely reliable, but is sufficient for seeds that have to be germinated as some viability tests, such as the analysis of protein synthesis, would result in the destruction of the seed.

Experimental design and treatments

Seed pre-treatment methods carried out were: soaking in 98% concentrated sulphuric acid, nicking/cutting, filing, boiling, hot water treatment, moist pre-chilling, moist pre-warming and a control in which the seeds were left intact.

The control

Thirty untreated seeds were sown in fifteen plastic pots. Each of the pre-treatments was replicated three times.

Seed scarification methods

Soaking in 98% concentrated sulphuric acid

Thirty seeds were placed in a 200 ml beaker and covered in 100 ml of 98% concentrated sulphuric acid. Seeds were gently stirred to soak and then removed after twenty minutes and washed thoroughly with distilled water before being planted.

Nicking/cutting

Thirty seeds had their tips cut cross-sectionally using a sharp knife, exposing the seed embryo. Care was taken to avoid cutting the seed embryo. The seeds were then immediately sown.

Filing

Thirty seeds had their tips filed using a file. This was done until a tiny hole appeared exposing the embryo before the seeds were planted.

Boiling

Thirty seeds were soaked in boiling water for 20 min.

Hot water treatment

Thirty seeds were soaked in boiled water overnight and planted after twenty hours.

Table 1. Mean number of germinated *B. discolour* seeds by various pre-treatments.

Method of pre-treatment	N (number of trials)	Mean germinated ± SE (*B. discolour*)
Filing	3	1.33±1.33[a]
Nicking/cutting	3	0.00± 0.00[b]
Sulphuric acid (98% concentration)	3	0.00± 0.00[b]
Hot water scarification	3	0.00± 0.00[b]
Boiling	3	0.00± 0.00[b]
Pre-chilling	3	0.00± 0.00[b]
Control	3	0.00± 0.00[b]
Filing and pre-chilling	3	0.00± 0.00[b]
Nicking and pre-chilling	3	0.00± 0.00[b]
Sulphuric acid and pre-chilling	3	0.00± 0.00[b]

[a,b]Means within the same column with different superscripts are significantly different at P<.05.

Seed stratification methods

Moist pre-chilling

Thirty seeds were embedded in 50 cm^3 of moist sand in a closed glass bottle. They were then stored in a refrigerator at 5°C for 5 days under regular monitoring of the setup to ensure that the medium remained moist. The seeds were remoistened during the period and planted.

Moist pre-warming

Thirty seeds were embedded in 50 cm^3 of moist sandy soil in a closed glass bottle stored at 60 to 70°C for 5 days. After this incubation period, the seeds were planted.

Seed treatment by both scarification and stratification

In one treatment, thirty seeds were filed and then pre-chilled before being planted. The other thirty nicked seeds were also pre-chilled and then planted. Thirty sulphuric acid scarified seeds were also pre-chilled and then planted. Thirty seeds were treated with boiling water for 20 min and were pre-chilled before being planted.

Planting

Planting was done in potted loam soil. Fifteen 100 cm^3 plastic pots were used per each treatment. In each pot, two holes of about 1 cm depth were drilled by fingers, and two seeds were sown. Watering was done three times a day using a fine spray and where necessary, weeding was performed. Sowing was done in a shaded well ventilated garden.

Data analysis

Data were statistically analyzed using SPSS version 22.0. The effects of the pre-treatment methods on *B. discolour* seed germination were tested using LSD post-hoc tests at the P<0.05 level of significance.

RESULTS

Filing was the only successful pre-treatment methods found to break dormancy of *B. discolour* seeds and result in germination. All the other pre-treatments failed to germinate the seeds within a period of 28 days (Table 1).

Seeds that had been cut had empty embryo cavities and seeds from all the other pre-treatments were found intact. This implies that seeds were still viable and dormancy had not been broken.

DISCUSSION

There was a significant difference (p<0.05) between filed seeds and seeds by other pre-treatments in which filing of the seeds managed to break dormancy and resulted in marginal germination. The mean germination rate of 13.3% is too low and makes this investigation inconclusive.

Seeds pre-treated by boiling, hot scarification, pre-chilling and all the others were recovered and observed. These were found intact and undamaged. It therefore shows that the seeds may have remained in the soil dormant and still viable. Since *B. discolour* trees are commonly found on termite mounds, termites (*Isoptera* spp.*)* may be involved in breaking seed dormancy. The termites may also fetch the seeds from sites of dispersal

and bring them to the mounds. Termites eat the hard seed coat and even the embryos of the *B. discolour* seeds. However, some termites may eat the seed coats and leave the seeds without damaging the embryos. This process could allow germination to take place, as the seed coats will be weak.

Results show that weakening of the seed coat by opening a tiny hole to the cavity holding the embryo enhances germination. The three methods of seed scarification used which include filing, cutting and soaking in sulphuric acid, which help weaken the seed coat, should have resulted in germination. However, cutting of the seed coat to expose the embryo resulted in no germination. It may be suggested, that the process of cutting may have damaged the embryo in the process resulting in its failure to germinate. Seed germination influenced by sulphuric acid is due to its capability to rupture seed coat enhancing absorption and thus imbibition of seeds. For seeds scarified by sulphuric acid, failure to germinate may also be attributed to the duration spent by the seeds in the concentrated acid, which may have been too short to cause enough weakening of the seed coats or the duration may have been long enough to cause damage to the embryo thereby failing germination (Salisbury and Ross, 1992; Ali at al., 2011).

When seeds are treated by boiling, the intention is to open up holes on the seed coat closed by plugs. This is achieved by increasing internal seed pressure as noted by Salisbury and Ross (1992). Failure by the seeds to germinate after boiling treatments may indicate that *B. discolour* seeds have no strophiolar clefts and plug as in some seeds. Strophiolar clefts are plugs covering special openings present in the seed coats which enhance germination by being loosened or removed to allowing water into the seed (Bewley and Black, 1994). Boiling therefore does not result in the germination of *B. discolour* seeds as it does not affect the hard coat. High temperatures may have a negative effect on the germination of *B. discolour* seeds. It can be speculated that, boiling could have killed the embryo and thus prevented any chance of germination.

Stratification did not achieve germination in *B. discolour* seeds. Low temperatures are indicated in Whyman (1992) as causing damage to *B. discolour* seeds. Cold and moist warm treatments did not soften the seed coats. After the 28 days in the soil, the seeds were observed intact with their seed coats still very hard. The subjection to cold may have damaged the embryo and resulted in no germination (Whyman, 1993). Seed treatments with hot water had been described to improve germination of hard seed coat species by uplifting water and oxygen permeability of the testa of seed coat (Ali at al., 2012). In this study, cold and hot water seed stratification failed to encourage *B. discolour* seed germination. Permeability of the seed coat to water was not improved by stratification. Seeds that had double pre-treatment were initially scarified and later stratified. No germination was achieved from any of the double treatments. Pre-chilling of seeds that had initially been filed could have damaged the embryo and failed seed germination.

Naturally, it is suggested that *B. discolour* seeds germinate by breaking of the barrier to water and gases provided by the hard seed coat. This might be achieved by exposing the seed-exocarp to attack by ants or alternate wetting and drying. These mechanisms achieve dormancy breakage in teak trees as noted by Davison and Fairlamb (1976). Our results provide evidence that *B. discolour* seeds enter into dormancy after maturation in their fruits. The seeds' germination is dependent upon mechanisms that break dormancy.

Conclusion

Results of this study show that methods of scarification, particularly filing considerably influences germination of *B. discolour* seeds. Similar studies are therefore recommended to improve the effect of filing and other scarification methods on breaking seed dormancy in *B. discolour.* Improvements on the other pre-treatment methods should be made in later investigations to verify findings of this study. Research should be intensified to investigate the physiology of seed germination and dormancy of indigenous species to help identify suitable pre-treatments to break dormancy which should result in high germination rates within short periods. Attention to propagation of indigenous species which are under extinction threats due to over harvest and land degradation is recommended.

Conflict of Interest

The authors have not declared any conflict of interest.

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