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Contents

Bacillus megaterium (AUM72)-mediated induction of defense related enzymes to enhance the resistance of turmeric (*Curcuma longa* L.) to *Pythium aphanidermatum* causing rhizome rot

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ABSTRACT

Bacillus megaterium (AUM72),a plant growth promoting rhizobacteria (PGPR) isolated from the rhizosphere soil were evaluated for their ability to control rhizome rot in turmeric(*Curcuma longa* L).Under in vitro condition, *B.megaterium* (AUM72), showed maximum inhibition of mycelia growth of *Pythium aphanidermatum*, were found effective in reducing rhizome rot of turmeric both under greenhouse and field conditions and increased the plant growth and rhizome yield. This isolate was further tested for its ability to induce production of defense related enzymes and chemicals in plants. Increased activities of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase,chitinase and β-1,3 –glucanase were observed in *B. megaterium* (AUM72) pre-treated turmeric plants challenged with *P.aphanidermatum*. Higher accumulation of phenolics was noticed in plants pre-treated with B.megaterium (AUM72) against *P.aphanidermatum*. Thus the present study shows that in addition to direct antagonism and plant growth promotion, induction of defense –related enzymes involved in the phenyl propanoid pathway collectively contributed to enhance resistance against invasion of *Pythium* in turmeric.

Key words: *Bacillus megaterium*, *Pythium aphanidermatum*, induced resistance, peroxidase

INTRODUCTION

India, "the land of spices" is the largest producer, consumer and exporter in the world. Turmeric (*Curcuma longa* L.) stands next to black pepper and cardamom. Turmeric is susceptible to diseases, such as leaf blight, anthracnose and rhizome rot. Among the various diseases, rhizome rot caused by *Pythium* sp. is a major problem in all turmeric-growing areas of India (Rathiah 1987, Nageshwar Rao 1994, Ramarethinam and Rajagopal 1999). The increased reflection on environmental concern over pesticide use has been instrumental in a large upsurge of biological disease control. Development of fungicide resistance among the pathogens, ground water, foodstuff pollution and the development of oncogenic risks have further encouraged the exploitation of potential antagonistic microflora in disease management. Although fungicides have shown promising results in controlling the damping-off disease, phytotoxicity and fungicide residues are major problems leading to environmental pollution and human health hazards. Development of fungicide resistance by *Pythium* sp. further discourages its use for disease control (Whipps and Lumsden 1991).

Bacillus species are the most common bacteria isolated from the soil, which accounted up to 36% of the bacterial population. *B. subtilis* is bestowed with the ability to endure stress, which is an advantage over other root colonizing bacteria. This bacterium also has the ability to produce antifungal metabolites and antibiotics to exploited for the control of many plant pathogens. Huang et al. (1999) reported that *B. licheniformis* and *Trichoderma* spp. effectively controlled Cucumber seedling damping off caused by *P. aphanidermatum* in soil less culture. *Bacillus* sp. effectively controlled *P. aphanidermatum* causing seedling disease of Okra (Anitha and Tripathi 2001). Seed bacterisation of Tomato with *B. subtilis* FZB 44 significantly reduced the yield loss caused by *P. aphanidermatum* (Maloupa and Gerasopoulos 2001). Individual strain of *B. subtilis* varied significantly with the fungal species and host crop. In Tomato, the highest efficacy against *P. debaryanum* was obtained with Bs 30.Induced systemic resistance (ISR) by definition refers to protection of the plants systemically by enhancement of plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate inducing agent upon infection by a pathogen. Induction of systemic resistance by PGPR against various diseases considered as the most desirable approach in crop protection. ISR once

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expressed activates multiple potential defense mechanisms that include increased activity of chitinases, β-1,3-glucanases and peroxidases (Dalisay and Kuc 1995, Xue et al. 1998) and accumulation of antimicrobial low molecular weight substances phytoalexins (Van Peer et al. 1991, Van Peer and Schippers 1992) and formation of protective biopolymers, e.g. lignin, callose and hydroxy proline-rich glycoprotein (Hammerschmidt et al. 1982).

The objectives of the present study are (1) To evaluate the efficiency of *B. megaterium* AUM72 against *P. aphanidermatum* under in vitro conditions and against rhizome rot under greenhouse and field conditions. (2) To study the induction of various defense-related enzymatic activities implicated in response to infection by *P. aphanidermatum* and by treatment with *B.megaterium* (AUM72).

MATERIALS AND METHODS

Cultures used

The plant growth promoting rhizobacteria *Bacillus megaterium* AUM72 was obtained from the Department of Microbiology, Faculty of Agriculture, Annamalai University. The isolate has been maintained in NA slants at 30± 2°C with monthly transfer. The test pathogen *p. aphanidermatum* from the department of Microbiology, Faculty of Agriculture, Annamalai University, this isolate was maintained in Potato Dextrose Agar.

Field experiment

Efficacy of *B. megaterium* AUM72 against rhizome rot disease

Two field experiments were conducted in two villages, viz. Trial I at Azichigudi village in Cuddalore District and Trial II at Oruthur village in Cuddalore District, Tamil Nadu, India, where the rhizome rot disease is endemic. Rhizomes were treated with 10 g of *B. megaterium* AUM72 formulation ($x10^{-6}$) per turmeric seed kg. As a standard treatment, rhizomes were treated with 6 g of Ridomil per turmeric seed kg. Seeds treated with distilled water served as control. The talc-based formulations were applied to soil after third and fifth MAP to enhance the population of antagonistic bacteria in the soil. The experiment was laid out with randomized block design with four replications. The plant height, rhizome rot incidence and rhizome yield were recorded at the time of harvest.

In vitro experiment

Induction of defense mechanism and experimental design

The following treatments took place in a greenhouse. The humidity in the greenhouse was maintained at around RH 80%. The temperature was adjusted to 26°C (day)/20°C (night). 3 replicants were maintained in each treatment. Each replicant was composed by 8 pots. The experiments were conducted using randomized block design on a greenhouse bench. (1) rhizomes treated with *B. megaterium* AUM72; (2) rhizomes treated with *B. megaterium* AUM72 and challenge with *P. aphanidermatum* 30 days after planting (50 g sand–maize medium containing10^3 cfu g^{-1} medium in each pot); (3) plants inoculated with the pathogen 30 days after sowing; and (4) non-treated plants. Rhizomes were sown in earthen pots filled with sterilized potting soil at rate of three rhizomes per pot. Plants were carefully uprooted without causing any damage to rhizome tissues at different time intervals (0, 2, 4, 6, 8 and 10 days after the pathogen inoculation). Three plants were sampled from each replication of the treatment separately and were maintained separately for biochemical analysis. Fresh rhizomes were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized rhizome tissues were stored at -70°C.

Table 1: *Bacillus megaterium* AUM72 to efficacy on inhibition of *P. aphanidermatum* under *in vitro* condition

Treatment	Mycelial growth(cm)	Germination (%)	Vigour index
Control	00.00	35.00	630.00
B.megaterium	36.11	85.66	1950.10

The percentage data were arcsine transformed prior to the analysis. In a column, means followed by a common letter are not significantly different (p=0.05) by Duncan' Multiple Range Test.

Assay of peroxidase activity

One gram of fresh plant tissue was ground in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar The homogenate was centrifuged at 15,000 x g at 48°C for 15 min. The supernatant was used as enzyme source. 0.1 ml from the obtained enzyme extract was added to 1.5 ml of 0.05 M pyrogallol and 0.5 ml of 1% H_2O_2 to obtained the reaction mixture. The change in absorbance of the reaction mixture was recorded at 420 nm at 30-s interval for 3 min at room temperature (28°C)**.** The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in absorbance of the reaction mixture per minute per gram of rhizome (Hammerschmidt et al. 1982).

Assay of polyphenol oxidase

One gram of fresh plant sample was ground in 1 ml of 0.1 M sodium phosphate buffer (pH = 6.5). The homogenate was centrifuged at 15,000 x g for 15 min at 48°C and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 and 0.1 ml of the enzyme extract. The reaction

was initiated by the addition of 0.2 ml of catechol (0.01 M). The activity was expressed as change in absorbance at 495 nm at 30-s interval for 3 min. The enzyme activity was expressed as change in absorbance per minute per gram of rhizome (Mayer et al. 1965).

Assay of phenylalanine ammonia lyase

One gram of rhizome was homogenized in 5 ml of cold 25 mM borate HCl buffer (pH = 8.8) containing 5 mM mercaptoethanol. The homogenate was centrifuged at 15,000 x g for 15 min and the supernatant was used as enzyme source. The assay mixture consists of 0.2 ml of enzyme extract, 1.3 ml water and 0.5 ml borate buffer. The reaction was initiated by the addition of 1 ml of 12 mM L-phenylalanine. The reaction mixture was incubated for 1 h at 32°C. The reaction was stopped by the addition of 0.5 ml of 2 N HCl. A blank was run in which phenylalanine was added after adding 2 N HCl. The absorbance was measured at 290 nm. The enzyme activity was expressed as µmol of cinnamic acid/min/g of rhizome (Dickerson et al. 1984).

Estimation of phenols

One gram of the rhizome sample was ground in a pestle and mortar in 10 ml of 80% methanol. The homogenate was centrifuged at 10,000 x g for 20 min. The supernatant as evaporated to dryness and the residue was dissolved in 5 ml of distilled water. From this, 0.2 ml was taken and the volume was made up to 3 ml with distilled water. To that 0.25 ml of Folin-Ciocalteau reagent (1 N) was added. After 3 min, 1 ml of 20% sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was measured at 725 nm against a reagent blank. The phenol activity was expressed in mg of catechol per gram of plant tissue (Meena and Mathur 2003).

Assay of chitinase

Rhizome samples (1 g) were homogenized in 2 ml of 0.1 M sodium citrate buffer (pH = 5.0). The homogenate was centrifuged at 16,000 x g for 15 min at 48°C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 10 ml of 1 M sodium acetate buffer (pH = 4.0), 0.4 ml of enzyme extract and 0.1 ml of colloidal chitin (10 mg). After 2 h incubation at 37°C, the reaction was stopped by centrifugation at 8000 x g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 ml of 1 M potassium phosphate buffer (pH = 7.1) and incubated with 20 ml desalted snail gut enzyme (Helicase). Finally, the mixture was incubated with 2 ml of dimethyl amino benzaldehyde for 20 min at 37°C and the absorbance was measured at 585 nm. The enzyme activity was expressed as nmol GlcNAc equivalents per minute per gram Rhizosphere (Bakkar et al. 2003).

Assay of β-1,3-glucanase

β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan et al. 1991). Rhizome samples (1 g) were extracted with 2 ml of 0.05 M sodium acetate buffer (pH = 5.0) and centrifuged at 16,000 x g for 15 min at 48°C. The supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 ml of4% laminarin and 62.5 ml of enzyme extract. The reaction was carried out at 40°Cfor 10 min. The reaction was then stopped by adding 375 ml of dinitrosalicylic acid and heating for 5 min on boiling water, vortexed and its absorbance was measured at500 nm. The enzyme activity was expressed as mg glucose released minute per gram rhizome (Pieterse et.al 2001).

Statistical analysis

All the experiments were repeated once with similar results. The data were statistically analyzed (Gomez and Gomez 1984) and Duncan's multiple range test compared treatment means. The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

RESULTS

The recent survey in different turmeric growing areas of Tamil Nadu, recorded rhizome rot incidence ranging from 20 to 50% incidence. The pathogens responsible for causing rhizome rot were isolated and identified as *P. aphanidermatum*, *R. solani* and *Fusarium oxysporum*. Pathogenicity studies on the involvement of pathogen in causing disease were studied. It was observed that *P. aphanidermatum* (49.33%), *R. solani* (14.33%) and *F. oxysporum* (9.33%) were found to be associated with rhizome rot of turmeric. Among them, *P. aphanidermatum* was found to be more virulent than *R. solani* and *F. oxysporum* in causing rhizome rot of turmeric.

Based on the in vitro studies on the inhibition of mycelial growth of *P. aphanidermatum*, isolate such as *B. megaterium* AUM72 was able to inhibit the mycelial growth of *P.aphanidermatum* under in vitro conditions. Under greenhouse condition *B.megaterium* AUM72 recorded 30.20% rhizome rot incidence followed by control inoculated with *P. aphanidermatum* recorded a rhizome rot incidence of 72.66%. The efficacy of *B. megaterium* is comparable with that of chemical Ridomil (Table 2).

The efficacy of talc-based formulations of *B. megaterium* AUM72 against rhizome rot was tested under field conditions. Formulations of *B. megaterium* AUM72 were effective for the control of rhizome rot incidence and increased plant growth under field conditions studied in two locations. The efficacy was comparable with chemical treatments.

Table 2: Efficacy of *Bacillus megaterium* (AUBM29) against rhizome rot disease of turmeric under green house conditions

Treatments	Rhizome rot incidence (%)	Height (cm)	Yield (g/plant)
Bacillus megaterium	30.00	75.50	450.00
Pathogen inoculated	72.55	50.10	250.00
Bm+pathogen inoculated	32.20	74.20	430.00
Control (water)	0.00	54.00	320.00
Ridomil (chemical)	33.40	70.40	360.00

Effect of rhizobacteria on the induction of defense- related enzymes and phenolic compounds against rhizome rot of turmeric

The activity of peroxidase (PO) increased in *B. megaterium* AUM72 treated rhizome tissues challenged with the pathogen. The maximum PO activity was observed on the fourth day after challenge inoculation and the activity was maintained at higher levels throughout the experimental period. Plants inoculated with the pathogen alone had comparatively less PO activity. PO activity in rhizomes treated with *B. megaterium* AUM72 alone remained unchanged during the experimental period but compared with control, the activity was higher (Figure 1(a)).

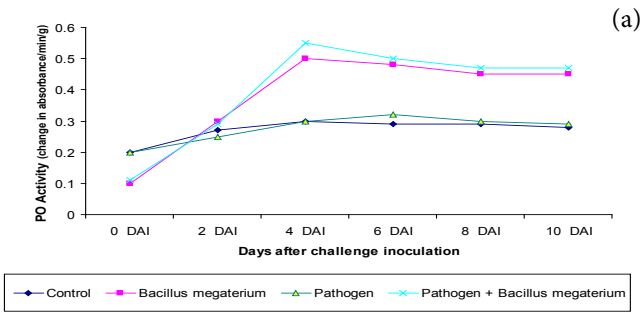
A similar pattern of increased activity of PPO was observed in bacterised turmeric plants challenged with the pathogen and the activity reached maximum on the fourth day after challenge inoculation (Figure 1(b)). The activities of chitinase (Figure 1(c)) and β-1,3-glucanase (Figure 1(d)) were maximum in bacterised rhizome challenged with the pathogen and higher activities were observed at sixth and fourth days after challenge inoculation, respectively.

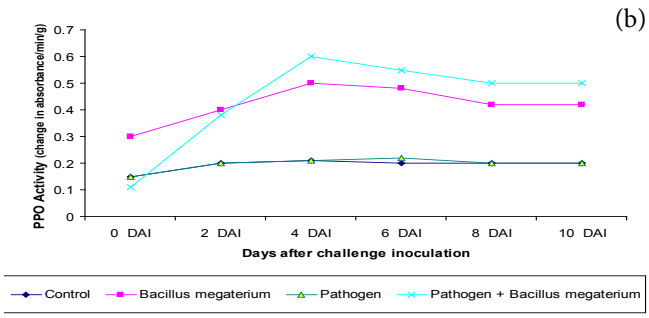
Enzyme activity

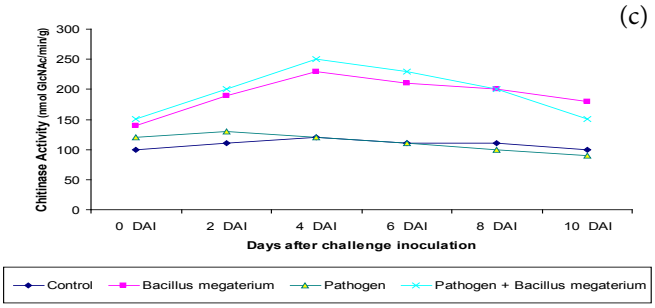
Generally, the enzyme activities were the maximum in bacterised rhizomes challenged with the pathogen. Induction of these enzyme activities was observed two days after challenge inoculation in bactericide plants. In plants inoculated with the pathogen alone, the enzyme activity increased initially at 4 day but later declined drastically at 8 day.

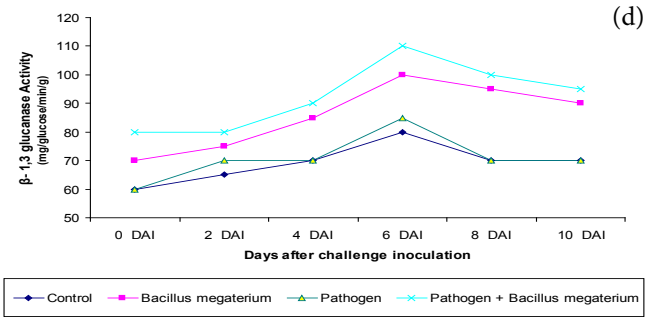
On pathogen challenge in bacterised turmeric tissues, phenylalanine ammonia lyase (PAL) activity started to increase on the second day after the pathogen inoculation and reached maximum on the sixth day after the pathogen challenge. Similarly, turmeric rhizomes inoculated with the pathogen alone-recorded increased activity of PAL but the induction of activity was observed for 2–4 days, there after declined drastically.

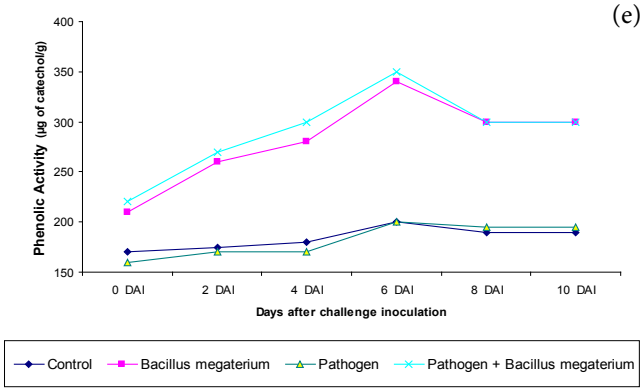
Turmeric plants treated with *B. megaterium*AUM72 alone also had significantly higher PAL activity compared











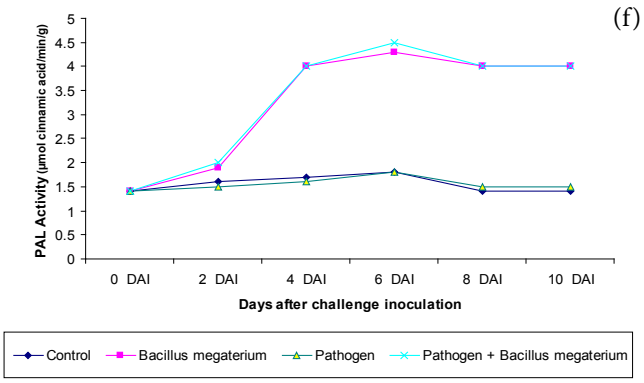


Figure 1: (a) changes in PO activity by rhizome treatment with *B.megaterium* and in turmeric challenged with and without *P. aphanidermatum.*(b) changes in PPO activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum* (c) Induction of Chitinase activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum*. (d) Induction of β-1,3 glucanase activity by rhizome treatment with *B. megaterium* and in turmeric challenged with and without *P. aphanidermatum*. (e) Influence of rhizome treatment with *B. megaterium* and on PAL activity in turmeric challenged with and without *P. aphanidermatum* (f) Accumulation of Phenolics by of rhizome treatment with *B. megaterium* in turmeric challenge with or without *P. aphanidermatum.*

with untreated control but activity was less compared with challenge-inoculated plants (Figure1(e)).

Defense mechanism

Studies on induction of defense mechanisms revealed that higher accumulation of phenolics was observed in *B. megaterium* AUM72 bacterised turmeric rhizomes challenge inoculated with *P. aphanidermatum*. Accumulation of phenolics started second day after challenge inoculation**.** The maximum accumulation was phenolics activity observed on the sixth day (350) after challenge inoculation. Plants inoculated with the pathogen alone also recorded increased accumulation of phenolics, but accumulation started on the second day after pathogen inoculation and drastically declined days after inoculation. Moreover, the accumulation of phenolics was less compared with bactericide plants challenged with the pathogen (Figure 1(f)).

DISCUSSION

Bacillus species is the major potential unexploited biocontrol agent against soil borne*of P. aphanidermatum* causing rhizome rot of turmeric. The inhibition of pathogen might be due to the production of antifungal metabolites. Production of antibiotics such as Iturin, Bacillomycin, Zwittermycin A and Surfactin is responsible for their antifungal action (Silo-Suh et al. 1994, Asaka and Shoda 1996, Constantinescu 2001, Moyne et al. 2001, Hiradate et al. 2002).

The present study also indicates that application of talc-based formulation of *B. megaterium* AUM72 increases plant growth in the both field experiments and reduced disease incidence. The results are in agreement with the findings of Meena and Mathur (2003) that treating ginger seed rhizomes with bioagents combined with soil application could effectively suppress the rhizome rot of ginger and increase the yield. Previous studies of McKeen et al. (1986) and Abdelzaher (2003) report the successful application of *B. subtilis* to control seedling diseases on a number of crops such as against a number of rhizome and seedling pathogens.

The increase in the hydrolytic enzymes in turmeric plants via challenge inoculation of AUM72 might help in defending the ingress of *Pythium* into host system. Kloepper at al. (1991) reported that ISR induced by fluorescent *Pseudomonas* was associated with induction of chitinase, as a promising technology for the management of red rot of sugarcane. In the present study, the activity of chitinase and β-1,3-glucanase was higher in biocontrol agents pre-treated plants challenge inoculated with *P. aphanidermatum*, which might have resulted in the lysis of invading pathogen. PO and PPO catalyse the last step in the biosynthesis of lignin and other oxidative phenols. Increased activity of cell wall bound PO has been elicited in different plants such as cucumber (Chen et al. 2000), rice (Reimers et al. 1992). In bean rhizosphere, colonisation of various bacteria induced the PO activity (Zdor and Anderson 1992). In the present study, PO activity was significantly increased from second day after challenge inoculation. The PO activity was elevated on fourth day after challenge inoculation in turmeric rhizome. Radjacommare (2000) reported that *P. fluorescens* isolate Pf1 induced the activities of PPO in rice against *R. solani*. Similarly, Meena et al. (2000) reported that the *P. fluorescens* induced the activities of PPO in response to infection by *C. personata* in groundnut. Similarly, significant increase in the activity of PPO was observed in green gram plants treated with P. fluorescens along with chitin (Saravanakumar 2002). Increases in PO activities were detected in foliar extracts of tomato whose seeds had been microbiolised, with Rhizobacterium B101R, against *Alternaria solani* (early blight), *Corynespora cassiicola* (foliar blight), *Oidium lycopersici* (powdery mildew), *Stemphilium solani* (leaf spot) and *Xanthomonas campestris* pv. *vesicatoria* (bacterial spot) of tomato (Silva et al. 2004). In the present study, higher PO activity were detected in *B. megaterium* AUM72 treated plants challenge inoculated with *P. aphanidermatum*.

In the present study, treatment with B. megaterium AUM72 induced the plants to synthesise PAL when the plants were challenge inoculated with *P. aphanidermatum*. The activity reached the maximum level on sixth day after challenge inoculation in turmeric rhizome pre-treated with the AUM72 which was challenged with *P. aphanidermatum*. Invasion of rhizome tissues by the pathogen might have resulted in decreased activity of

Table 3: Efficacy of talc based formulation of *Bacillus megaterium* (AUBM 29) on the management of rhizome rot of turmeric under field conditions

Treatments	Trial-1			Trail-2		
	Rhizome rot incidence (%)	Plant height (cm)	Yield (g/plant)	Rhizome rot incidence (%)	Plant height (cm)	Yield (g/plant)
Bacillus megaterium	29.00	85.50	436.00	30.00	86.00	490.40
Control	52.00	63.10	296.00	51.00	62.20	310.10

PAL whereas earlier and increased activity of PAL due to treatment with *B. megaterium* AUM72 might have prevented fungal invasion and thus the activity was maintained at the higher levels. When groundnut plants were sprayed with P. fluorescens, increased activity of PAL was observed (Meena et al. 1999, 2000).

Increased activity of PAL was observed in *P. fluorescens*-treated tomato, pepper, mint, rice and ragi plants in response to infection by *F. oxysporum* f. sp. *lycopersici*, *C. capsici* and *R. solani* (Meena et al. 1999, Ramamoorthy et al. 2002a, Radjacommare et al. 2005).

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, rhizome treatment with *B. megaterium* (AUM72) resulted in increased accumulation of phenolic substances in response to infection by the pathogen. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al. 1996). Benhamou et al. (1996) reported that an endophytic bacterium, *Serratiaplymuthica* induced the accumulation of phenolics in cucumber rhizomes following infection by *P. ultimum*, *P. fluorescens* Pf1 isolate also induced the accumulation of phenolic substances and PR-proteins in response to infection by *F. oxysporum* f. sp. *lycopersici* in tomato (Ramamoorthy et al. 2002a), *Pyricularia grisea* in ragi (Radjacommare et al. 2005).

CONCLUSIONS

In conclusions, the present study implies that earlier and higher accumulation of enzymes involved in phenylpropanoid metabolism and PR-proteins has been found in turmeric rhizome tissue treated with *B. megaterium* AUM72 in response to invasion by *P. aphanidermatum*. The plant-pathogen interactions have also triggered the activities of defense enzymes initially but later the activities drastically declined when the pathogen colonised the root tissues. Accumulation of phenolics, PAL, β-1,3-glucanase and induction of *B. megaterium* (AUBM29) in turmeric rhizome tissues might have collectively contributed to induced resistance in turmeric plants against *P. aphanidermatum.*

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Effect of varying level of diversification and intensification of rice-wheat cropping system on the production potential and nutrient balance of soil in Indo-Gangetic Plain of India

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ABSTRACT

Since few decades, continuous practice of Rice-Wheat Cropping System (RWCS) has been led to depletion of inherent soil fertility and thereby productivity, resulting in a serious threat to its sustainability in the Indo- Gangetic Plain Region (IGPR) of India. Unfortunately, farmers in the region rarely introduce legumes in the systems which have great significance to restore fertility and productivity of soil. Keeping these aforesaid facts in view, a long-term field experiment initiated during 2000-01 to assess the impact of rice based cropping sequences. The data of the experimental year 2002-03 and 2003-04 showed that addition of one more crop in rice-wheat cropping system either summer grain/ fodder legumes or Sesbania (green manure) resulted higher Economic Rice Yield Equivalent (EREY), protein yield, nutrient uptake vis a vis increased soil nutrient balance due to improvement in physico-chemical properties of soil. Among different cropping system under study, rice-potato-green gram gave highest EREY whereas highest protein recovery and nutrient (N, P and K) uptake were associated with rice-maize (cob) + veg. pea (1:1)-cowpea (fodder). Conclusively, Rice-potato-green gram and rice-maize (cob) + veg. pea (1:1)-cowpea (fodder) appeared promising alternatives to replace the rice-wheat cropping system in IGPR.

Key word: cropping system, rice equivalent yield, soil productivity, nutrient balance, diversification and intensification

INTRODUCTION

The introduction of short duration, photo-insensitive, dwarf and input responsive high yielding varieties of rice and wheat in the mid-1960s has led to adoption of rice-wheat cropping system (RWCS) in India and more particularly in the entire Indo-Gangatic Plain Region (IGPR). In recent years, the productivity of this sequences decreases drastically (Anon 1991) due to the degradation of soil fertility by the intensive cereal-cereal production systems and is considered a major cause of the decline yield in the region. A rice-wheat system yielding 7 t ha^{-1} of rice and 4 t ha^{-1} of wheat removes 315 kg nitrogen (N) ha^{-1}, 28 kg phosphorus (P) ha^{-1} and 333 kg potassium (K) ha^{-1}, and significant amount of micronutrients (Hegde and Dwivedi 1992). Therefore, for sake of food security, farmers are being compelled to apply higher rates of the fertilizers every years in incremental fashion (particularly N fertilizer) to maintain the same yield levels as it was attained previous year with less fertilizer use.

During the rice-wheat cropping cycle, soil under goes drastic change *i.e.* anaerobic to aerobic environment, leading to several chemical and electro-chemical transformations. Besides the contrasting need of each crop, continuous practice of rice-wheat cropping system for longer period with least system diversity and often with poor crop management practices, resulted in loss of soil fertility due to emergence of multiple nutrient deficiency (Singh and Singh 1995), deterioration of soil physical properties (Tripathi 1992) and decline in factor productivity and crop yields in high productivity areas (Yadav et al. 1998). Certainly, refinement of nutrient management strategies helps to maintain the crop productivity and soil fertility, but other rotational strategies could help especially in situation where greater fertilizer use may be uneconomic or environmentally unacceptable. In this manner, a nutrient cycling mechanism that is essential for the sustainability of the production system can be established. Inclusion of pulses, oilseeds and vegetables in the system is more beneficial than cereal after cereal (Raskar

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and Bhoi 2001). Introduction of a legume crop in RWCS may have advantages well in the improvement in physic-chemical properties of soil, beyond the N addition through biological nitrogen fixation (BNF) including nutrient recycling from deeper soil layers, minimizing soil compaction, increase in soil organic matter, breaking of weed and pest cycles and minimizing harmful allelopathic effects (Sanford and Hairston 1984, Wani et al. 1995). Soil organic carbon and available N, P and K increased markedly, when the wheat in rice-wheat cropping system was substituted with a legume (Hegde and Dwivedi 1992). Growing of legumes as green manure can provide the equivalent of 60 kg of urea N ha^{-1} to following rice with little or no residual effect on a succeeding wheat crop (Kolar and Grewal 1988). Mandal and Chatterji (1998) documented that N, P and K uptake by crops and soil fertility improved significantly due to inclusion of legume as green manure before transplanting of rice in the rice based cropping sequences. In addition, it is also reported that legumes are favorable for increasing the yield of succeeding rice crops (Quayyam and Maniruzzaman 1996). As much as 10% of the rotation effect on grain yield of a subsequent cereal may be due to N benefits from the legume crop in the rotation (Stevenson and Van Kessel 1996). Hargrove (1986) estimated that contribution of legume cover crop as source of N to subsequent non leguminous crops may be as high as 120 kg N ha^{-1}. In another long term experiment at Saskatchewan, Campbell et al. (1991) reported increasing soil organic matter content with increasing frequency of cropping and especially inclusion of legumes as green manure in the rotation. On the other hand, Soil N content under crop rotation is increased in two major ways: N input as atmospheric fixation during rotation of legume and secondly reducing leaching loss due to increasing soil organic matter (Arshad et al. 1998).

Most of the micro and macro level previous studies on cropping systems in the IGPR have focused mainly on specific aspects of rice-wheat cropping system. Studies providing an integrated assessment of more diversified intensive double and tripal cropping systems have remained relatively less studied but such studies are needed for understanding options for diversification and intensification of the rice-wheat system in IGPR. The present investigation was therefore undertaken to study the system production potential, soil nutrient balance as well as changes in physico-chemical properties of soil under varying levels of intensification and diversification in traditional rice-wheat cropping system.

MATERIAL AND METHODS

A long term field experiment entitled "Effect of diversification and intensification of rice-wheat cropping system on the productivity and nutrient balance of soil in Indo-Gangetic Plain" was initiated under All India Coordinated Research Project on Cropping Systems during 2000-2001 at Agricultural Research Farm, Banaras Hindu University, Varanasi, India but data of experimental year 2002-03 and 2003-04 only compiled for current research paper.

Varanasi is situated at 25^0 18' N latitude, 83^0 03' E longitude and at an elevation of 128.93 m. It falls under middle Gangetic plain zone of the IGPR. The climate is semi arid to sub humid with hot dry summer and cold winters. The soil was sandy loam and classified as Ustochrep. The top soil (0-15 cm layer) at the start of experiment was non saline (EC 0.34 dS m^{-1}) of neutral reaction (pH 7.3) and contained 0.34 % organic carbon, 192.0 kg ha^{-1} available N, 21.4 kg ha^{-1} available P and 224.0 kg ha^{-1} available K. The treatments consisting of ten crop sequences were arranged in four randomized blocks. The crop sequences were:

1. S_1: Rice (*Oryza sativa* L.)-wheat (*Triticum aestivum* L.)
2. S_2: Rice-chickpea (*Cicer arietinum* L.)
3. S_3: Rice-wheat-green gram (*Vigna radiata* L.)
4. S_4: Rice-wheat-*Sesbania* (*Sesbania aculeata* L.) for green manuring
5. S_5: Rice-mustard (*Brasica juncea*)-green gram
6. S_6: Rice-lentil (*Lens culinaris* L.)-cowpea fodder (*Vigna unguiculata* L.)
7. S_7: Rice-pea (*Pisum sativum* L.)
8. S_8: Rice-lentil+mustard (3:1)-cowpea fodder
9. S_9: Rice-maize (*Zea mays* L.) for green cob+vegetable pea (1:1)-cowpea fodder
10. S_{10}: Rice-potato (*Solanum tuberesum*)-green gram

The details of the varieties used and cultural operations adopted in different crop sequences are given in Table 1. The gross plot size was 7 x 6 m with one meter plot border. With a view to avoid the mixing of soil in different treatments, individual plots were thoroughly prepared by power tiller in each season. Before sowing of any legumes component, they were treated with appropriate strain of *Rhizobium spp*.as seed treatment. *Sesbania aculeata* as green manure and green gram after last picking were cut from the ground level and green biomass so obtained was weighed and incorporated *in situ*. The cowpea for green fodder was harvested from the ground level. The rest of the crop was harvested at maturity. However, harvesting of maize for green cob, vegetable pea and cowpea for green fodder was done at proper stage.

To study changes in soil fertility, initial soil samples were collected at beginning of the experiment (2000) with auger from 0-15 cm soil depth at 20 locations of the experimental area as per standard procedure. The samples were thoroughly mixed, dried and passed through 100 mesh sieve and kept in poly bags for chemical analysis of organic carbon (rapid titration method), pH (1: 2.5 soil : water), available N (alkaline permanganate method), available P (0.5N $NaHCO_3$ extractable) and available K (Flame photometric method). Soil samples were also analyzed treatment-wise at the completion of each cycle. The changes in soil organic carbon and available N, P and K in different crop sequences were worked out on the basis of initial soil nutrient status and nutrient status of soil at termination of the experiment (2003-04).

Representative samples of harvested grain/ seed/ cob/ tuber and straw/ stover/ fodder were also taken for chemical analysis. The plant samples were successively wash thoroughly with tap water, 0.05M HCl solution and deionized water, and dried at 70^0 C in a hot air oven. The dried samples were milled in a stainless steel Wiley mill and wet- digested in a 4:1 mixture of nitric and perchloric acid, and aqueous extracts prepared for the determination of total P Spectro-photometrically (using vandomolybdate yellow colour method), and of total

Table 1: Describing different systems with level, rate and desired state

Crop in rotation	Cultivar	Seed rate (kg ha^{-1})	Spacing (cm)	Date of sowing 2002-03	Date of harvesting 2002-03 2003-04	Rate of fertilizer application(kg ha^{-1})		
						N	P	K
2002-03								
Rice	NDR 97	30	20	15 July 02	14 Oct 02	120	60	60
Wheat	HUW 468	100	20	25 Nov 02	4 April 03	120	60	60
Chickpea	Avarodhi	80	30	14 Nov 02	28 March 03	18	46	20
Mustard	PRO 4001	5	45	14 Nov 02	16 March 03	90	45	45
Lentil	Pant 209	40	30	14 Nov 02	22 March 03	18	46	20
Pea(grain)	HUP 15	80	30	14 Nov 02	22 March 03	18	46	20
Veg. pea	Arkel	80	25	15 Nov 02	15/26 Feb 03	18	46	20
Maize(cob)	Decan 103	20	75	15 Nov 02	16 April 03	120	60	40
Potato	Kufari Badshah	2000	50	15 Nov 02	16 March 03	120	60	80
Green gram(S_3)	Jyoti	25	30	8 April 03	6 July 03*	18	46	0
Green gram (S_5)	Jyoti	25	30	26 March 03	21 June 03*	18	46	0
Green gram(S_{10})	Jyoti	25	30	26 March 03	21 June 03*	18	46	0
Sesbania(GM)	-	60	-	-	-	0	0	0
Cowpea (F) (S_6)	Local	40	30	2 April 03	28 June 03	18	46	0
Cowpea (F) (S_8)	Local	40	30	2 April 03	28 June 03	18	46	0
Cowpea (F) (S_9)	Local	40	30	18 April 03	28 June 03	18	46	0
2003-04								
Rice	NDR 97	30	20	25 July 03	13 Oct 03	120	60	60
Wheat	HUW 468	100	20	27 Nov 03	8 April 04	120	60	60
Chickpea	Avarodhi	80	30	13 Nov 03	2 April 04	18	46	20
Mustard	PRO 4001	5	45	13 Nov 03	18 March 04	90	45	45
Lentil	Pant 209	40	30	13 Nov 03	18 March 04	18	46	20
Pea(grain)	HUP 15	80	30	13 Nov 03	18 March 04	18	46	20
Veg. pea	Arkel	80	25	14 Nov 03	13/24 Feb 04	18	46	20
Maize(cob)	Decan 103	20	75	14 Nov 03	9 April 04	120	60	40
Potato	Kufari Badshah	2000	50	14 Nov 03	12 March 04	120	60	80
Green gram(S_3)	Jyoti	25	30	16 April 04	29 June 04*	18	46	0
Green gram (S_5)	Jyoti	25	30	30 March 03	15 June 04*	18	46	0
Green gram(S_{10})	Jyoti	25	30	23 March 03	11 June 04*	18	46	0
Sesbania(GM)	-	60	-	-	-	0	0	0
Cowpea (F) (S_6)	Local	40	30	5 April 04	30 June 04	18	46	0
Cowpea (F) (S_8)	Local	40	30	5 April 04	30 June 04	18	46	0
Cowpea (F) (S_9)	Local	40	30	17 April 04	30 June 04	18	46	0

*Green gram after last picking the biomass cut from the ground level and incorporated *in situ.*

K content (Flame photometrically). For the determination of N, the ground material was digested separately in a 4:1 mixture of H_2SO_4 and $HClO_4$ and analyzed by the micro Kjeldahl method (Piper 1966). Protein content in economic produces of different crops was estimated by multiplying 6.25 with nitrogen content in economic produces of respective crop.

The productivity of different crop sequences was compared by calculating their Economic Rice Equivalent Yield (EREY) using formula given by Ahlawat and Sharma (1993). Where,

$$\text{EREY} = \frac{\text{Yield of each crop (t ha}^{-1}\text{) x Economic value of respective crop (Rs. t}^{-1}\text{)}}{\text{Price of rice grain (Rs. t}^{-1}\text{)}}$$

For this the price of individual output were assumed to be stable during the experimental period. The economic values of the produce from different crops were taken as Rs. 6500.0 t^{-1} for rice, Rs. 10000.0 t^{-1} for wheat, Rs. 27000.0 t^{-1} for chickpea, Rs. 20000.0 t^{-1} for mustard, Rs. 22000.0 t^{-1} for lentil, Rs. 19000.0 t^{-1} for field pea, Rs. 4500.0 t^{-1} for maize (cob), Rs. 6000.0 t^{-1} for vegetable pea, Rs. 4000.0 t^{-1} for potato tuber, Rs. 32000.0 t^{-1} for green gram and Rs. 800.0 t^{-1} for cowpea fodder.

The data were analyzed statistically by analysis of variance (Cochran and Cox 1957) to ascertain statistical differences between cropping systems, and level of significance was

calculated by two way ANOVA and expressed as $p < 0.05$.

RESULTS AND DISCUSSION

As evident from table 2, in the rainy season, crop sequences involving summer grain/ fodder legume or *Sesbania* for green manuring tended to be greater rice yield than rice grown in rice - wheat cropping system. However, the differences were significant only 2003-04. As the experiment was initiated in 2000 - 2001, it took four years to become legume effect significant in terms of rice yield. The crop sequences, *viz.* S_4, S_8, S_{10}, S_9 and S_3 though remained at par among themselves, produced significantly greater grain yield of rice than rice-wheat system. In winter season, potato out yielded other crops and the next best was maize (cob) + vegetable pea (1:1) as intercropping. This could be ascribed to their high production potential and harvesting of the economic produce fresh at high moisture contents. Among three sequences involving wheat as winter crop, the greater wheat yield was recorded in rice-wheat-*Sesbania* (5.68 and 9.58%) followed by rice-wheat-green gram (1.8 and 7.25%) over rice-wheat system during 2002-03 and 2003-04, respectively. This is due to improvement in soil physico-chemical properties (Table 5) in these treatments by inclusion of *Sesbania* as green manuring (S_4) and templing of green gram after last pecking. Sharma et al. (1995) also advocated the benefit of green manure before the rice in rice-wheat system for enhancing the wheat yield. Out of the two summer crops that are green gram and cowpea fodder grown in different crop sequences, green gram performed better when taken after potato (S_{10}) and mustard (S_5) than after wheat in S_3. This was mainly due to timely sowing of green gram after potato and mustard (Table-1) that provided favorable weather conditions for initial growth and development as well as sufficient period for three picking before onset of mansoon. Whereas, only two picking were possible in green gram taken after wheat due to delayed sowing of green gram (S_3). In contrast, there was not much variation in fodder yield of cowpea taken in different sequences *viz.* S_6, S_8 and S_9. This indicates that timely sowing of summer green gram is more important than that of cowpea fodder. Considering system wise REY, sequences having three crops each year produced significantly greater REY than rice-wheat system during both the years. The highest REY was recorded from rice-potato-green gram rotation possibly due to besides, higher production potential of potato, higher grain yield of green gram after potato were instrumental for attaining maximum REY than rest of the sequences. The rice-maize (cob) + veg. pea (1:1)-cowpea (fodder) and rice-mustard-green gram sequences ranked second and third respectively, recorded significantly greater REY than rice-wheat. This might be due to higher production potential of maize (cob) along with good market price of vegetable pea and mustard that fetched better returns of these two sequences. Green gram in S_3 markedly contributed to system besides enhancing the productivity of succeeding crops and consequently resulted in significantly higher REY than rice-wheat system (S_1). The improvement of soil structure following legume (Wani et al. 1995), breaking of cycle of the pest and diseases and allelopathic effect of residue of legume crops (Sanford and Harison 1984) may be additional reason for extra yield from these sequences.

The crop sequences with summer grain/ fodder legumes resulted better protein recovery (Table 3), produced 808.8 and 1369.6 kg ha^{-1} in 2002-03 and 983.8 and 1427.4 kg ha^{-1} in 2003-04, it was significantly greater than the protein production under traditional rice-wheat cropping system, produced 708.1 and 726.8 kg ha^{-1} in 2002-03 and 2003-04, respectively. Besides direct contribution of summer legume in total system protein production, its residual effect on succeeding rice, obviously improved its yield and N uptake (Table 2 and 4) due to nitrogen being an important component of protein resulted greater protein production. The maximum protein yield of rice-maize (cob) + veg. pea (1:1)-cowpea (fodder) rotation was due to higher biological yield of maize as well as inclusion of two leguminous crop *i.e.* vegetable pea and cowpea fodder, in the sequence. Tripathi et al. (1987) also reported intensification of crop sequences with pulses considerably increased the production of protein. However, the poor performance of chickpea and pea (Table 2) caused lower protein production of rice-chickpea and rice-pea sequences.

In general, sequences involving summer grain/fodder legume or *Sesbania* as green manure, one extra crop taken during summer season of experiment results greater N, P and K uptake by sequences during both the years of experimentation (Table 4). The N uptake of the sequences having cowpea fodder *viz.* S_6, S_8 and S_9, were higher than the sequences *viz.* S_3, S_5 and S_{10} comprising green gram. This was due to fact that in green gram only pods were picked up but for cowpea fodder, the entire crop was cut at the ground level. Nevertheless, all the crop sequences having summer green gram, cowpea fodder or *Sesbania* for green manuring resulted significantly higher nutrient (N, P and K) removal than standard rice-wheat cropping system. Here it may be pointed out that in present investigation, besides the direct contribution of green gram/cowpea fodder in nutrient uptake, it improved the productivity (Table 2) and nutrient concentration in component crops in respective sequence were responsible for this result. However, *Sesbania* as green manure in rice-wheat-*Sesbania*, not directly involved in nutrient uptake, but it enhanced the productivity and nutrient content of component crops, which resulted in higher nutrient uptake of this sequence. This result corroborates the findings of Chandrasekharan and Sankaran (1995). Sequence S_9 removed highest amount of N, P and K, and proved significantly greater N and P uptake than other sequences but in respect to K uptake it remained at par to all the triple cropping systems. This could be ascribed mainly to its maize (cob) + vegetable pea (1:1) intercropping with good productivity. Similarly, due to lentil + mustard (3:1) intercropping, sequence S_8 was next to S_9 and significantly higher N and P uptake than other sequences. Due to poor performance of chickpea and pea, the rice-chickpea and rice-pea sequences recorded significantly lower nutrient uptake than rice-wheat system, indicating major cause of decline in fertility of soil under traditional rice-wheat system.

Soil pH declined in all the sequences except rice-wheat, which remained increase from initial 7.30 to 7.43. The maximum reduction in pH value was recorded under rice-

Table 2: Economic yield and economic rice equivalent yield (t ha^{-1}) of different cropping systems

Treatments	Rice crop		Winter crops		Summer crops		Economic rice equivalent yield	
	2002-03	2003-04	2002-03	2003-04	2002-03	2003-04	2002-03	2003-04
S_1- Rice-Wheat	3.60	4.01	3.87	3.86	-	-	9.53	9.95
S_2- Rice-Chickpea	3.64	4.12	0.83	1.19	-	-	7.06	9.06
S_3- Rice-Wheat-Green gram	3.73	4.46	3.94	4.14	0.75	0.62	13.47	13.89
S_4- Rice-Wheat-*Sesbania* (GM)	3.84	4.59	4.09	4.23	-	-	10.13	11.10
S_5- Rice-Mustard-Green gram	3.85	4.26	1.29	2.12	0.95	1.06	12.52	16.02
S_6- Rice-Lentil-Cowpea (F)	3.92	4.34	1.05	1.01	24.30	23.56	10.47	10.65
S_7- Rice-Pea	3.75	4.27	1.48	1.16	-	-	8.06	7.67
S_8- Rice-Lentil+ Mustard (3:1)-Cowpea(F)	3.76	4.55	0.69 (0.67)*	0.62 (1.04)	23.52	24.66	11.05	12.89
S_9- Rice-Maize (cob)+ veg. Pea (1:1)-Cowpea (F)	3.83	4.53	9.22 (2.01)	9.87 (1.99)	24.65	24.04	14.67	16.16
S_{10} - Rice-Potato-Green gram	3.85	4.53	17.77	23.66	1.27	1.25	21.02	25.23
SEm ±	0.12	0.13	-	-	-	-	0.35	0.42
CD (P = 0.05)	N.S	0.38	NA	NA	NA	NA	1.01	1.23

*Figures in parentheses shows the value in intercrop, SEm = Standard Error of Mean and CD = Critical difference = Least significant difference

Table 3: Protein yield (kg ha^{-1}) from economic produce of different crop sequences

Treatments	Rice		Winter crops		Summer crops		Total system	
	2002-03	2003-04	2002-03	2003-04	2002-03	2003-04	2002-03	2003-04
S_1- Rice-Wheat	277.8	305.0	430.3	421.9	-	-	708.1	726.8
S_2- Rice-Chickpea	288.8	327.3	173.1	241.4	-	-	461.9	568.7
S_3- Rice-Wheat-Green gram	299.9	361.4	449.0	470.3	180.9	152.0	929.8	983.8
S_4- Rice-Wheat-*Sesbania* (GM)	315.2	382.7	471.7	483.9	-	-	787.0	866.6
S_5- Rice-Mustard-Green gram	312.3	345.0	266.5	428.1	230.0	254.8	808.8	1027.8
S_6- Rice-Lentil-Cowpea (F)	326.0	365.3	228.0	221.2	699.2	682.3	1253.2	1268.8
S_7- Rice-Pea	297.7	341.9	297.3	235.5	-	-	595.0	577.4
S_8- Rice-Lentil+ Mustard (3:1)-Cowpea (F)	305.1	373.5	287.8	341.6	666.6	706.4	1259.5	1421.5
S_9- Rice-Maize (cob)+ veg. Pea (1:1)-Cowpea (F)	313.4	372.8	355.9	368.2	700.3	686.4	1369.6	1427.4
S_{10} - Rice-Potato-Green gram	303.3	356.7	444.2	563.3	302.2	298.2	1049.7	1218.2
SEm ±	11.0	11.5	11.8	21.7	25.0	17.7	25.5	31.2
CD (P = 0.05)	NS	33.3	34.1	62.8	75.3	53.3	73.9	90.7

Table 4: Total N, P and K uptake (kg ha^{-1}) by different crop sequences

Treatments	N		P		K	
	2002-03	2003-04	2002-03	2003-04	2002-03	2003-04
S_1- Rice-Wheat	173.45	176.91	28.69	29.46	182.29	185.70
S_2- Rice-Chickpea	130.19	158.90	17.38	20.81	116.90	138.63
S_3- Rice-Wheat-Green gram	211.25	225.77	32.95	35.62	200.73	223.50
S_4- Rice-Wheat-*Sesbania* (GM)	192.21	208.77	32.35	34.94	213.46	228.47
S_5- Rice-Mustard-Green gram	199.34	244.81	29.93	37.78	188.37	218.40
S_6- Rice-Lentil-Cowpea (F)	272.35	268.98	36.04	35.82	215.46	211.16
S_7- Rice-Pea	163.97	150.06	21.05	20.04	142.93	135.40
S_8- Rice-Lentil+ Mustard (3:1)-Cowpea(F)	277.00	300.70	38.48	42.36	227.70	233.58
S_9- Rice-Maize+ Pea (1:1)-Cowpea (F)	329.54	332.00	46.03	45.57	229.96	228.36
S_{10} - Rice-Potato-Green gram	208.56	248.03	33.48	39.83	195.26	216.67
SEm ±	5.33	7.03	0.82	0.86	6.15	5.2
CD (P = 0.05)	15.46	20.39	2.39	2.49	17.85	16.88

Table 5: Physico-chemical properties of soil and nutrient balance after four year completion of experiment under different crop sequences

Treatments	pH	BD (Mg m^{-3})	O. C. (%)	Available N (kg ha^{-1})	Available P (kg ha^{-1})	Available K (kg ha^{-1})	Nutrients balance (kg ha^{-1})		
							N	P	K
S_1- Rice-Wheat	7.43	1.46	0.34	186.2	21.70	194.2	-5.8	0.3	-29.8
S_2- Rice-Chickpea	7.29	1.43	0.36	200.4	24.70	209.4	8.4	3.3	-14.6
S_3- Rice-Wheat-Green gram	7.00	1.34	0.37	206.1	22.20	218.7	14.1	0.8	-5.3
S_4- Rice-Wheat-*Sesbania* (GM)	6.97	1.31	0.39	218.6	23.20	219.5	26.6	1.8	-4.5
S_5- Rice-Mustard-Green gram	7.03	1.35	0.38	205.4	22.80	216.2	13.4	1.4	-7.8
S_6- Rice-Lentil-Cowpea (F)	7.06	1.33	0.37	212.5	23.00	211.6	20.5	1.6	-12.4
S_7- Rice-Pea	7.26	1.42	0.35	199.8	22.00	207.3	6.8	0.6	-16.7
S_8- Rice-Lentil+ Mustard (3:1)-Cowpea (F)	7.06	1.35	0.38	216.4	22.30	207.6	24.4	0.9	-16.4
S_9- Rice-Maize (cob)+ veg. Pea (1:1)-Cowpea (F)	7.05	1.34	0.38	211.2	22.50	201.4	19.2	1.1	-22.6
S_{10} - Rice-Potato-Green gram	6.98	1.35	0.37	203.3	22.40	204.6	10.3	1.0	-19.4
SEm ±	0.11	0.03	0.01	5.41	1.20	9.08	-	-	-
CD (P = 0.05)	0.31	0.08	NS	15.70	NS	NS	NA	NA	NA
Initial value	7.30	1.44	0.34	192.0	21.4	224.0	-	-	-

NA- Not analysed

wheat-*Sesbania* (green manure) sequence and though it remained at par to the sequences involving summer green gram or cowpea fodder *viz*. S_3, S_5, S_6, S_8, S_9 and S_{10}, all registered significantly lower soil pH than rice-wheat cropping system (Table 5). This decline in soil reaction might be due to organic compound added to the soil during decomposition of green as well as root biomass, produced more humus and organic acids. The CO_2 and organic acids produced during decomposition help in mobilizing calcium by dissolving calcium compound and consequently lower down the pH

(Bajwa 2002).

The sequences involving summer grain/fodder legume or *Sesbania* for green manure, significantly reduced bulk density of soil than rice-wheat system (Table-5). The lowest bulk density of soil was recorded in rice-wheat-*Sesbania* (green manure) rotation. Green manuring is an age old practices, adds lot of carbonaceous material and relatively narrow C/N ratio resulting lower down the bulk density and improves the physical condition of soil (Chhonkar and Pareek 2002).

In respect to organic carbon content (%) and available N (kg ha^{-1}) in soil at the termination of experiment, both were highest in rice-wheat-*Sesbania* (green manure) rotation (Table-5). The other sequences *viz.* S_8, S_6, S_9, S_3, S_5, and S_{10}, though remained at par to rice-wheat-*Sesbania* (S_4), recorded significantly greater organic carbon and available soil N than traditional rice-wheat cropping system. This may be attributed to the biomass added in the soil through green manuring by *Sesbania,* incorporation of green gram stover and roots as well as root and stubbles of cowpea fodder. Besides this, the sequences having summer green gram, cowpea fodder or *Sesbania* for green manuring, improved plant growth and ultimately yield of component crops in respective sequences (Table 2). It was quite obvious that this might have added greater root biomass and stubble to the soil consequently improving soil organic carbon. Campbell et al. (1991) advocated that organic matter content increased with increasing frequency of cropping and inclusion of legume as green manure crop in the rotations. This improved organic carbon accelerated nitrogen fixation by free living organism. The sequences having winter legume (S_2 and S_7) also showed slightly more available N in the soil, because of its biological nitrogen fixation ability. These results support by earlier studies (Ahlawat et al. 1981, Deka and Singh 1984). Another reason for improved available soil N is rotations containing legume crop reduces losses of soil N in the form of NO_3 leaching (Campbell et al. 1997).

As compared to rice-wheat system, sequences involving summer grain/fodder legume or *Sesbania* for green manuring, recorded greater available P and K at the end of the field trial. However, differences were not significant. This might be due to during decomposition of organic matter various organic acid are produced, solublized the phosphate and potassium bearing minerals. The incorporation of green manure also reduces P sorption by soil (Tiwari 2002).

At completion of four years experiment, the positive balance of soil N in all the sequences was observed except in rice-wheat system, where negative balance of N (- 5.8 kg ha^{-1}) was noticed. This could be ascribed to the excessive removal of N by rice-wheat system (Table 4) accompanied with low efficiency of applied N in spite of recommended dose of N applied to both the crops. The positive balance of soil available P was observed in all the sequences as compared to initial value. This might be due to comparatively lower uptake of P by crops (Table 4) than it application. When each component crop of an intensive production system receives P at the recommended rate, the apparent P balance remained positive in most growing situation (Swarup and Wanjari 2000). The higher positive soil available P balance was recorded in rice-wheat-*Sesbania* (green manure) and sequences having two leguminous crops. Amending soil with green manures, help in increasing P concentration in soil solution through mineralization of organic P and solubilization of native soil P compound resulted greater available P balance in the soil (Tiwari 2002). Contrary as compared to initial value, the available K balance of soil found to be negative in all the rotations after four crop cycle. This was mainly due to heavy removal of K by all the crop sequences (Table 4). The summer legumes in the present study did not receive fertilizer K, and K application to other crops were not sufficient to meet demand (Singh et al. 2002). The maximum K deficit was recorded in rice-wheat system. This result was also supported by earlier work of Srivastava and Srivastava (1993).

CONCLUSIONS

Inclusion of summer crop beneficially augmented soil physical as well as chemical properties of soils and productivity of the systems as whole. Substitution of rice-wheat with triple cropping system particularly rice-potato-green gram or rice-maize (cob) + vegetable pea (1:1)-cowpea (fodder) sequences, not only produce relatively higher rice equivalent yield, but also increased protein production. Diversification/ intensification of rice-wheat with summer grain/fodder legumes or *Sesbania* as green manuring, decreased soil pH, bulk density and improved the soil organic carbon as well as available N and P status of the soil.

Based on the experimental findings, it is recommended that intensely and purely grown rice-wheat cropping system in Indo-Gangetic Plain Region of India should be replaced with cropping system like rice-potato-greengram or rice-maize (cob) + vegetable pea (1:1)-cowpea (fodder) as these cropping systems are environmentally safe and economically viable. Hence, it would be imperative to make concrete and intensive efforts for creating awareness among farmers of IGPR of India regarding significance of aforesaid cropping system. Consequently, intensification and diversification of rice-wheat cropping system may be served as a base to uplift the socio-economic conditions of the farmers belonging to IGPR of India in general and particularly the small and marginal farmers by augmenting the production per unit area and per unit time along with restoring soil fertility and improving quality of resource bases.

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Morphological and physiological changes during adventitious root formation as affected by auxin metabolism: Stimulatory effect of auxin containing seaweed extract treatment

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ABSTRACT

The formation of adventitious roots is a quantitative genetic trait regulated by both environmental (especially temperature, light, relative humidity) and endogenous factors (hormones, sugars, mineral salts and other molecules). A cutting removed from the stock plant normally undergoes various anatomical changes accompanied by alterations in metabolic activity and gene expression during the wound response and subsequent rhizogenesis. Rooting consist of different steps, each with its own hormone requirements. Over recent years, a multitude of models have been proposed to show how plant hormones and metabolites interact to control adventitious root formation and plant development. Phytohormones are directly involved in cell division and cell growth, and they indirectly interact with other hormones or metabolites. Auxin plays an essential role among phytohormones when regulating roots' development and has been shown to be intimately involved in the process of adventitious rooting. Researchers have developed different rooting treatments, either by examining the effects of auxin using short exposure to a solution with a high auxin concentration or by dipping in a rooting powder (auxin with talc). Over the past two decades, auxin-containing seaweed extracts have been recognised as promising biostimulants for adventitious root formation and plant development. The aim of this review was to summarize current knowledge associated with the anatomical and physiological aspects of adventitious root formation, and highlight any recent progress made regarding the identification of auxin-containing seaweed extracts for the control of adventitious rooting.

Key words: adventitious root formation, auxin, carbohydrates, jasmonate, phenolics, proteins, seaweed extract

Abbreviations: hpe – hours post excision; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; NAA - 1-naphthalene acetic acid; PAL - phenylalanine ammonia-lyase; TAL - tyrosine ammonia-lyase; TIBA - triiodobenzoic acid;

ADVENTITIOUS ROOTS AND THEIR FUNCTION

The rooting of stem cuttings is one of the better and economically-viable propagation methods within agriculture, horticulture, and forestry. It is governed by an array of endogenous physiological factors (Druege and Kadner 2008, Osterc and Štampar 2008). Cuttings must survive physiological stress after severance from the stock plant, and if receiving little water or nutrient uptake they can be affected by the usually-reduced stomatal conductance, until the development of the roots (Druege and Kadner 2008, Pop et al. 2011). Consequently, chlorophyll loss and an increase in the xanthophylls cycle-pool are typically associated with leaf senescence. Further severance-induced metabolic responses include increased hormone production (auxin, ethylene, jasmonate) and establishment of a sink tissue, which is characterised by decreased endogenous carbohydrate status and the translocation of nutrients to the stem base for wound healing and cell division (Kadner and Druege 2004; Rapaka et al. 2005, 2008; Druege and Kadner 2008; Ahkami et al. 2009; Misra and Misra 2010; Trueman and Richardson 2011). Noticeable progress has been made recently during research into rooting, which is not a single process but a progressive process consisting of different steps, each with its own hormone requirements (de Klerk et al. 1999, Pop et al. 2011). Physiological characterization, histological analysis and molecular research on herbaceous and woody plants have recently provided a proper tool to distinguish these phases. Ahkami et al. (2009) postulated a three-phase mechanism for the metabolic response involved during adventitious root formation (ARF) on petunia used as model herbaceous plant. (1) The 'Sink establishment phase' is characterised by jasmonate (JA) accumulation immediately after wounding, and the induction of gene coding for enzymes that degrade

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sucrose within the apoplast into hexoses. The hexoses are taken up by at least one induced monosaccharide transporter and then used for the production of energy necessary for wound-healing and cell division. (2) The 'Recovery phase' is characterized by the replenishment of resources and in the petunia it lasts up to 72 hours after excision (hpe) with the formation of meristemoids. (3) The 'Maintenance phase' is characterized by the symplastic transport of sugars translocated from the source-leaves into the stem-base. They are converted, either into intermediates directly flowing into root development or to the intermediate storage compounds starch and citrate, thereinafter used for later root formation processes.

However, there is little information on rooting process in woody plants. As yet, molecular data are lacking and physiological studies are scarce. In apple microcuttings, the timing of three phases was ascertained (De Klerk et al. 1999). During the initial 24 hpe, dedifferentiation of the cells between the vascular bundles occurs from which the root primordial originate. These cells accumulate starch during the initial 24 hours. Wounding-related compounds (breakdown products of cell membranes, jasmonic acid, cellulase, pectinase) likely play a major role in the dedifferentitation phase, probably acting with auxins and cytokinins. Later on, up to 72 or 96 h, previously activated cells become commited to the formation of root primordial by the rhizogenic action of auxin. The starch grains are degraded during the following 24 h (24-48 hpe), the first cell divisions occur 48 hpe, and by 96 hpe, meristemoids are present. The meristemoids develop into root primordial and afterwards into roots during the differentitation phase. Indirect rooting of some species, in which root initials are formed after an intermediate callus phase, is an obvious example of the occurrence of three phases with a prolonged period of dedifferentiation. The differences in auxin requirements among the three phases of adventitious rooting have important consequences for rooting of cuttings (De Klerk et al. 1999). Taken together, ARF is modulated by a combination of different pathways, including hormone biosynthesis and primary metabolism; the individual steps exert a control over ARF. In the presented review, a spatio-temporal analysis of hormones and metabolites at different developmental stages of ARF is provided with respect to specific anatomical, hormonal and biochemical changes.

MORPHOLOGICAL CHANGES DURING ADVENTITIOUS ROOT FORMATION

On the basis of anatomical studies, the root formation phases were designated as the root initiation phase, followed by the root primordium formation phase, and then the root elongation and/or emergence phase. These anatomical events were studied precisely by Ahkami et al. (2009) on the model plant *Petunia hybrida* and by De Klerk et al. (1997, 1999) on apple microcuttings. After the severance of petunia cuttings from the stock plant, it took 72 h to initiate the earliest morphological event. This root initiation phase (Figure 1A) resulted in the occurrence of small cells with large nuclei and dense cytoplasms – both typical signs of meristematic cells. On the histological level, starch grains are degraded during the next 24 h, when the first cell divisions take place and meristemoids appear. The appearance of such meristemoids marks the transition from the root initiation phase to the root primordium formation phase (Figure 1B). This first cytological sign of ARF at 72 hpe was preceded by the RNA accumulation of the cyclin B1 gene, which might serve as a marker gene specific for the root initiation phase of ARF. During the root primordium formation phase, the first well-developed young root meristems became visible at 96 hpe (Figure 1C). At first globular structures developed root primordia with the typical dome-shape at 144 hpe, which included the meristem and behind it the first cells of the root body. The first roots with elongated cells of the elongation zone appeared at 192 hpe (Figure 1D). These structures were still inside the stem, but revealed all the morphology of a complete root, except for root hairs. They marked the transition to the root elongation phase, which resulted in the emerging of the earliest roots, visible after one additional day.

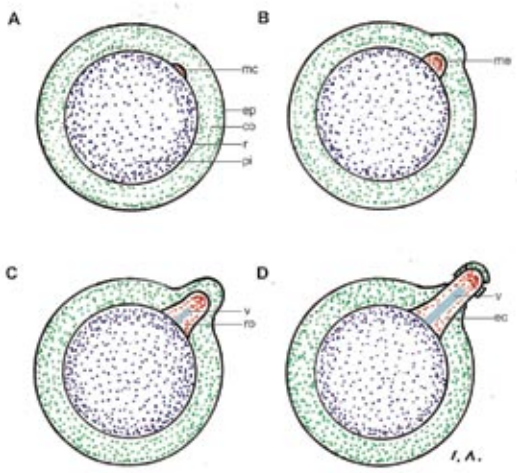

Figure 1: Anatomy of adventitious root formation (A-D) in the stem base of pelargonium (*Pelargonium peltatum*) cuttings. Typical stem anatomy consists of epidermis (ep), cortex (co), pith parenchyma (pi) and a ring of vessels (r) with outer phloem, the cambium, and the inner xylem. (A) „Root initiation phase": first meristematic cells (mc) of developing root meristems appear. (B) „Root primordium formation phase": the appearance of an meristemoid (me). (C) First differentiating root primordia with an organized meristem and a backward differentiation of cells of the root body containing root cortex (ro) and vascular bundle (v); (D) „Root elongation and/or emergence phase": emerging of the first root with vascular bundles (v) in the center surrounded by elongated cells (ec) of the elongation zone. It reaveals morphological characteristics of the complete root, except for root hairs.

HORMONE CONTROL AND METABOLIC RESPONSES INVOLVED IN ADVENTITIOUS ROOT FORMATION

Wound response and the role of jasmonates

The activation of wound-induced responses involves a complex network of signaling cascades, in which jasmonates (JA) represent the best-characterized class of signal molecules. Ahkami et al. 2009 reported that immediate after wounding, the JA content rose 12-fold and its precursor 12-oxo-phytodienoic acid (OPDA) had an approximately 4-fold accumulation, with a maximum 0.5 hpe. The JA contents returned to the basal level within 6 hpe, whereas OPDA remained at the increased level for 6 hours and dropped at 12 hpe to the basal levels. This time-course occurred locally within the stem base as well as systemically, but at lower levels, in other parts of the cuttings. Further analyses suggest that the protein constitutively present within vascular tissues has constantly high activity and is involved in the increased biosynthesis of JA, as already shown in the tomato (Hause et al. 2003, Stenzel et al. 2003). Usually the wound-induced elevation of JA levels is followed by the activation of JA-responsive genes (Wasternack and House 2002). In addition, cell wall-invertase transcript accumulation also seems to be induced by wound-induced JA within the stem base (Schaarschmidt et al. 2006). Higher levels of transcripts followed by an increased activity of cell wall invertase might then lead to a higher sink status of the stem tissue.

AUXIN BIOSYNTHESIS DURING ADVENTITIOUS ROOT FORMATION

Auxins are a group of tryptophan-derived signals that are involved in most aspects of plant development (Woodward and Bartel 2005). Auxin is mainly formed in young leaves and stem-tips, and is then transported to the roots, both in the phloem and by a special polar mechanism. Auxins play a major role in controlling the growth and development of plants, the early stages of embryogenesis, the organization of apical meristem (phyllotaxy) and the branching of the plant's aerial parts (apical dominance), formation of the main root, and lateral and adventitious root initiation (Went and Thimann 1937). Furthermore, it elicits those responses throughout the plant required for the function of developing leaves and roots. Auxins are also involved in gravitropism and phototropism (Kepinski and Leyser 2005). Auxin has a central role in shoot/root relation, correlating the presence and development of leaves with root initiation. In addition, auxin induces the differentiation of vascular tissues, it inhibits or induces the differentiation of branches and prevents the abscission of leaves (Sachs 2005, Pop et al. 2011). High photosynthesis could be coupled with auxin synthesis, thus enhancing root formation. High ion and water absorptions could be coupled with high auxin catabolism, thus enhancing leaf development (Sachs 2005). These multiple effects across the plant result from its control of cell division, cell elongation, and certain stages of differentiation. On the cellular level, the response to auxin includes a rapid initial cell-growth response that may involve auxin-induced changes in pH, calcium and gene expression (Davies 2004, Pop et al. 2011).

Rooting-phases have different auxin requirements. There is always a temporary increase in the endogenous level of free indol-3-acetic acid (IAA) during the inductive phase (corresponding to a minimal level of peroxidase/oxidase activity). The inductive phase is the auxin sensitive, when the plants are responsive to exogenous auxin application. It is followed by the auxin insensitive phase (initiation phase) characterized by a decrease in IAA levels to a minimum and high peroxidase and oxidase activity (Nag et al. 2001, Faivre-Rampant et al. 2002). The IAA oxidation at this stage of rooting (initiation phase) seems to be related to the auxin response. Oxidation products of IAA may promote root formation, especially when linked to the phenolic compounds present (Günes 2000). During the root expressive phase, IAA is again needed to promote the growth of root's initials (Štefančič et al. 2007).

The importance of auxin during the production of lateral or adventitious roots was demonstrated with several 'gain-of-function' as well as 'loss-of-function' *iaa* mutations (Fukaki et al. 2002, Rogg et al. 2001, Tatematsu et al. 2004). In *Arabidopsis*, the *superroot* (*sur1* and *sur2*) mutants, accumulate IAA, developing numerous adventitious roots on the hypocotyl and cuttings of different organs in the case of *sur1* (Delarue et al. 1998). Triiodobenzoic acid (TIBA), an auxin polar transport inhibitor, applied to the top of the hypocotyls, lowered the rate of root formation (Fabijan et al. 1981). Rice mutants affected in the expression of the *PIN-FORMED (OsPIN1)* gene, potentially involved in auxin polar transport, are affected in adventitious root emergence and tillering confirming that the auxin concentrations and distributions within the different tissues are essential (Xu et al. 2005).

New insight has been provided over recent years, into the interaction between auxin and other hormones. Auxin and ethylene are often described as activators, whilst cytokinins and gibberellins are seen as inhibitors of adventitious root formation, even when some positive effects have been observed. Auxin can increase the rate of ethylene biosynthesis (Riov and Yang 1989) and stimulate the production of ethylene correlating with the fact that the *ACC synthase4* gene has been found to be an early auxin-induced gene (Abel et al. 1995). The auxin and ethylene relations, during root development, has been shown by a number of isolated mutants that have resistance to both hormones (Müller et al. 1998, Pan et al. 2002). Studies have emphasized that polyamines play a role during adventitious rooting (Biondi et al. 1990, Hausman et al. 1994, Heloir et al. 1996) and a possible interrelation between polyamines and auxin-controlling rooting induction has been suggested (Hausman et al. 1995).

In addition over recent years, new insight into the interaction between auxin and carbohydrates, and their regulation of ARF, has been provided (Haissig 1989, Roitsch 1999, Druege and Kadner 2008, Agullo-Anton et al. 2011). It has been reported that auxin stimulates the mobilization of carbohydrates in leaves and the upper stem, and increases the translocation of assimilates towards the rooting zone.

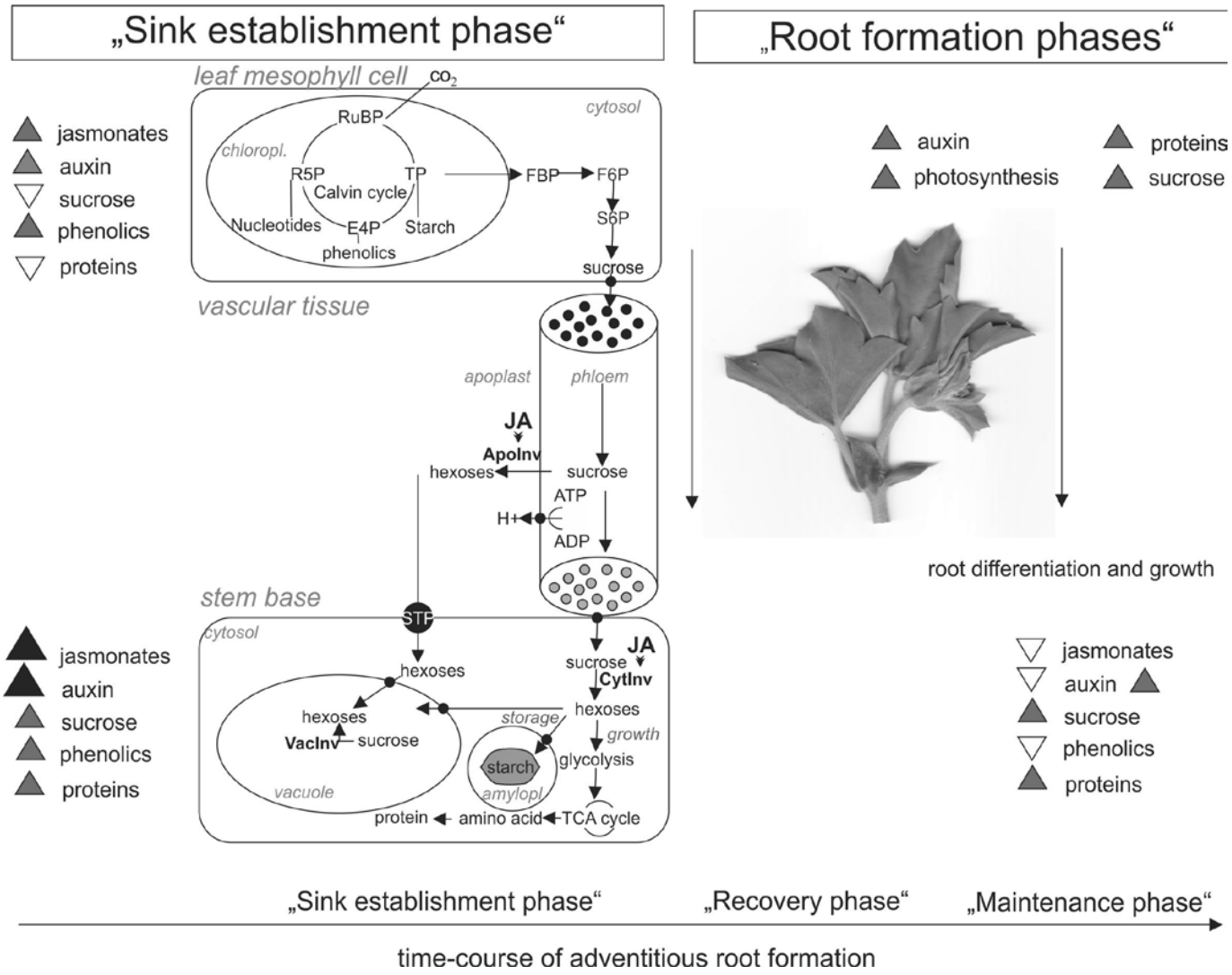

Figure 2: Schematic presentation of the metabolic response during adventitious root formation (ARF) based on model plants Petunia hybrida and Pelargonium peltatum (modified according to Ahkami et al. 2009, Urbanek Krajnc et al. 2012). Assimilates are produced in source tissues and translocated towards the cutting base to establish a sink organ. Wounding induces the accumulation of jasmonates (JA), which further induce the cell wall invertase (apoInv) transcript accumulation leading to cleavage of sucrose and transport of hexoses into the cell by monosaccharide transporter STP. Two days after excision the recovery starts followed by the maintenance phase characterised by symplastic transport of sugars from source leaves into the stem base and used either for energy production or accumulated in the vacuole. RuBP – ribulose-1,5-bisphosphate, TP – triose phosphate, E4P – erythrose-4-phosphate, R5P – ribulose-5-phosphate, FBP – fructose-1,6-bisphosphat, F6P – fructose-6-phosphate, S6P – sucrose-6-phosphate, apoInv – apoplastic invertase, cytInv – cytoplasmatic invertase, vacInv – vacuolar invertase, STP - monosaccharide transporter, TCA cycle – tricarboxylic acid cycle

THE IMPORTANCE OF CARBOHYDRATES IN ROOTING OF CUTTINGS

Cell division and cell enlargement during ARF require high input of energy and carbon skeletons. A major carbon source is sucrose, which is formed in photosynthetically active tissues and translocated towards the sink-parts of the plant. Sucrose can be used after cleavage into hexoses as a direct carbon source or is converted in to storage compounds such as starch (Figure 2). Moreover, there are increasing indications that sugars have a regulatory role in ARF (Takahashi et al. 2003, Gibson 2005).

In the stem-bases of petunia cuttings (Ahkami et al. 2009), the levels of soluble and insoluble sugars start to accumulate at 24 hpe, despite high metabolic activity from 12 to 24 hpe onwards. However, the most pronounced increase in sugar levels was documented during the later stages of ARF. A continuous increase of sucrose within source leaves can be observed from 144 hpe onwards, probably owing to an

increased photosynthetic capacity. This sucrose can be translocated towards the stem base for further metabolism. The basipetal translocated sucrose is, however, not only used for delivering energy for differentiation (cell division and cell enlargement), but considerable portion of the sucrose is converted into starch, which probably acts as the major carbon source when the adventitious roots are growing. As shown for *Pinus radiata*, sucrose applied to a growing-medium leads to higher levels of sugars and starch in the rooting regions of the hypocotyl cuttings, and enhances root formation (Li and Leung 2000). The analyses of Ahkami et al. (2009) indicated that starch can be synthesized and stored in different cell types in order to meet their demand for increased metabolic activity when the adventitious roots emerge (Ahkami et al. 2009).

In agreement with the low sucrose content, directly after excision (»sink establishment phase«), at the cutting base the cell wall invertase activity was increased during the first hours after excising, and decreased again to the basal level before root formation. By contrast, the activities of vacuolar and cytosolic invertases decreased continuously to a low level (Ahkami et al. 2009). Cell wall invertase is not only a key enzyme of the apoplastic phloem unloading of transported sucrose but also links phytohormone action with primary metabolism (Roitsch and González 2004). Being present within the apoplast, it can establish the sink function of a certain tissue and thus provide a mechanism for flexible and appropriate adjustment to a wide range of internal and external stimuli (Roitsch et al. 2003). The metabolic activity through which the hexoses from the basipetal transported sucrose can be catabolized leads to successive increases in both, phosphofructokinase (PFK) and glucose-6-phosphate dehydrogenase (Glc6P DH) activities, and a decrease in the cytosolic fructose-1,6-bisphosphatase activity. These events strongly suggest that the catabolism of glucose occurs in parallel within the pentose phosphate pathway and by glycolysis. This yields ATP as energy-source and amino acids for protein synthesis (Ahkami et al. 2009).

The importance of carbohydrates in ARF of pelargonium cuttings and the effects of carbohydrate shortage were studied by Druege and Kadner (2008). Especially, rooting of dark-stored pelargonium cuttings can be restricted by carbohydrate shortage resulting from the interplay between depleted carbohydrate reserves and weak photosynthesis under the low light conditions in the winter's in Central Europe. Druege and Kadner 2008 assumed that considerably reduced air temperature during rooting increased the current availability of carbohydrates, thereby improving survival and root formation. Higher sugar levels during rooting, mediated by low air temperature (10 °C, root zone temperature: 20 °C) were positively correlated with reduced leaf senescence, a higher survival rate, and a higher number of roots. The authors thus provided new prospects for the control of air temperature during the rooting of cuttings that exhibit low current photosynthesis.

AMINO ACIDS AND PROTEIN METABOLISM DURING ADVENTITIOUS ROOT FORMATION

In most of the plant systems analyzed, the most abundant amino acids in vascular tissues were glutamate, glutamine and, in some cases, asparagine (Urquhart and Joy 1982, Schobert and Komor 1989, Lohaus and Moellers 2000). Analysis of the petunia stems before excision indicated that mainly glutamine and asparagine were synthesized in the source-tissues and transported downwards to the sink-tissues (Ahkami et al. 2009). Since glutamine biosynthesis is the key step of nitrate assimilation in plants (Joy 1988) and supplies nitrogen for amino acid synthesis, the basipetal translocation of glutamine is an important process to meet the demands of the cells during root-formation. Levels of glutamine and asparagine remained low at the later stages. This indicated a high turnover of the translocated amino acids into others that appear to be essential for accelerated protein synthesis during ARF (Druege and Kadner 2008, Ahkami et al. 2009). A similar response was also determined for proteins in the case of pelargonium cuttings. In the presented experiment, a transient decline in total protein values was monitored twenty-three days after the severance of pelargonium cuttings (Urbanek Krajnc et al. 2012). However, this decline can not only be linked to the establishment of a sink-tissue and adequate mobilisation of primary metabolites to the region of root regeneration. Recent analyses of tomatos suggested that the proteins and amino acids constitutively presented in vascular tissues are involved in the increased biosynthesis of jasmonates, the best-characterised class of signal molecules involved in wound-induced responses (Hause et al. 2003). Jasmonate accumulation induces genes coding for invertases that degrade sucrose within the apoplast to hexoses used for the production of energy necessary for wound healing and cell devision. In the petunia stems' cuttings, the strong depletion of total amino acids between 6 and 48 hours after excision was measured and, thereafter, a recovery reaching only 50 % of the initial value was observed (Ahkami et al. 2009). These same authors confirmed a change of carbon to nitrogen ratio in petunias towards a threefold increase of carbon against nitrogen, beginning 48 hours post excision. The analysis of petunia stems pre-excision confirmed that mainly glutamine and asparagine were synthetised in source tissues and transported downwards to the sink tissues. Since glutamine biosynthesis is the key step of nitrate assimilation in plants and supplies nitrogen for amino acid synthesis (Taiz and Zeiger 2010), a basipetal translocation of glutamine is an important process for meeting the demands of the cells during root formation. These early events in ARF described by different authors would explain the observed depletion of protein contents within pelargonium cuttings 23 days post severance. Later on (36 dps), a strong increase was determined in the protein levels of pelargonium leaves (Urbanek Krajnc et al. 2012), which would be parallel to an increase in the carbohydrates due to high photosynthesis rates.

ROLE OF PHENOLICS DURING ADVENTITIOUS ROOT FORMATION

Severance from the stock plant, induces the biosynthesis of phenolic compounds, which is why many authors have detected an increased phenolic content level during the first days after establishing the cuttings (Osterc et al. 2004, Quaddoury and Amssa 2004, Štefančič et al. 2007, Osterc and Štampar 2008). Several results have indicated that some phenolic compounds (chlorogenic acid, epicatechin, caffeic acid, catechol, gallic acid, ferulic acid) act as rooting co-factors during the root formation process, especially as protectors of IAA against oxidation. On the other hand, the presense of specific phenolic compounds (sinapinic acid, vanillic acid, cinnamic acid) have a negative impact on ARF during the vegetative propagation of cuttings (Osterc et al. 2004, Trobec et al. 2005). Focusing on IAA protectors, two modes of action are ascribed: they might act as competing oxidation substrates for IAA-oxydase instead of IAA, or more likely they may function as free radical scavengers that drive the peroxidase-catalysed reaction (Trobec et al. 2005; Osterc et al. 2007, 2008; Štefančič et al. 2007; Osterc and Štampar 2008). Higher amounts of these phenolic substances mean more IAA in this way. Thus fluctuation of phenol contents during adventitious rhizogenesis undergoes a time-course variation paralleling that of free IAA, which is generally credited as the trigger for root initiation, and the physiological stages of rooting are correlated with changes in endogenous auxin concentrations (Bartel et al. 2001, Faivre-Rampant et al. 2002, Pop et al. 2011).

In the presented study, an increased accumulation of total phenolics three weeks after the establishment of pelargonium cuttings coincided with a decrease in total protein levels (Urbanek Krajnc et al. 2012). These results are in agreement with current knowledge that the synthesis of phenolics is coupled with amino acid metabolism and controlled by enzymes, including **phenylalanine** ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL). These enzymes act through the deamination of phenylalanine and tyrosine to yield t-cynnamic and p-coumaric acids, respectively, with the liberation of ammonia (Taiz and Zeiger 2010).

Thirty six days after severance, a slight decline in total phenolics was measured in pelargonium cuttings in comparison to the previous sampling (Urbanek Krajnc et al. 2012). A similar time-course of changes in phenolic contents has also been described by other authors (Trobec et al. 2005, Štefančič et al. 2007, Osterc and Štampar 2008). These authors first recorded increases in certain phenolic compounds in the leaves of chestnut cuttings, and the basal portions of cherry cuttings during rooting, later on the concentrations of monophenols rapidly decreased, especially sinapinic acid and vanillic acid, to which a negative influence on rooting is ascribed. Štefančič et al. (2007) reported that the fluctuation of phenolic content undergoes a time-course variation that parallels that of free IAA. A time-course analysis of single phenolic compounds and enzymes involved in the phenylpropanoid pathway would provide a better understanding of the interactions amongst indole-3-butyric acid (IBA) and phenolics during adventitous root formation in the future.

THE SIGNIFICANCE OF ENDOGENOUS AND EXOGENOUS AUXIN IN CONTROL OVER ADVENTITIOUS ROOT FORMATION

Auxin is one of the major endogenous hormones involved in the process of adventitious rooting and the physiological stages of rooting are correlated with changes in endogenous auxin concentrations. Plants produce IAA in the shoot apices and in young leaves. After detachment of the shoot, basipetal polar transport of auxin contributes to auxin accumulation in the stem base and the rise of free auxin in the basal stem very probably contributes to the early events of adventitious root formation (Ahkami et al. 2009). However, for successful rooting in difficult-to-root plant species, exogenous application of auxin can contribute significantly to the plant's auxin-pool and promote adventitious root formation (Pop et al. 2011). IAA was the first used to stimulate rooting of cuttings (Cooper 1935) and soon afterwards other auxins that also promoted rooting, IBA and NAA (1-naphthalene acetic acid) were synthesized chemically and were considered even more effective (Zimmerman and Wilcoxon 1935). Talc powder was introduced as a carrier for auxin (Grace 1937).

Nowadays IBA is used for rooting during commercial operations, followed by IAA and NAA, and the chemical analogues synthesized and examined for auxin-like activities. Auxin mostly enters cuttings via the cut surface (Kenney et al. 1969), even in microcuttings that are known to have a poorly functioning epidermis (Guan and De Klerk 2000), and is rapidly taken up in cells by pH trapping (Rubery and Sheldrake 1973) and by influx carriers (Delbarre et al. 1996). Auxin metabolism studies on adventitious rooting have been done on cuttings exposed to auxin over a prolonged period, but in other studies cuttings have been exposed to auxin over short periods (Diaz-Sala et al. 1996, Liu and Reid 1992) It was recognised, that an optimal auxin concentration for one of the three phases may be supraoptimal or suboptimal for the next. Over recent years, a multitude of models have been proposed for showing how auxin interacts for controlling adventitious root formation (ARF) and plant development (Sachs 2005, Jaillas and Chory 2010, Agullo-Anton et al. 2011, Pop et al. 2011). Although roots may be induced by auxin, it must be taken into a account that wounding is usually required to achieve rooting and it was suggested that WRCs (wounding-related compounds) play a major role during the de-differentiation phase (de Klerk et al. 1999).

When applying exogenous IBA on cuttings, the endogenous auxin concentration reaches a peak after wounding, thus coinciding with the initation of the rooting process. Interaction between endogenous IAA and exogenous IBA during ARF has been suggested and the performance of IBA versus IAA explained regarding several possibilities: higher stability, differences in metabolism, differences in transport, and IBA as a slow release source of IAA. Biochemical studies in numerous plants, and genetic studies of *Arabidopsis* IBA-mutants, indicate that IBA acts primarily via its conversion to IAA, which occurs through a mechanism similar to peroxisomal fatty acid b-oxidation; however, some evidence suggests that IBA acts as an auxin on its own (Pop et al. 2011).

The applied auxins are reported to lower the IAA oxidation, and this might reduce the consumption of phenolic antioxidants, which play a very important role as protectors of the IAA against oxidation (Volpert et al. 1995, Krylov et al. 1995, De Klerk et al. 1999)

Štefančič et al. (2007) investigated the influence of exogenous indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) on changes in the internal levels of IAA, indole-3-acetylaspartic acid (IAAsp) and antioxidant phenolics (chlorogenic acid and epicatechin) during the first 5 days of adventitious root formation in leafy cuttings of the cherry rootstock 'GiSelA 5'. The highest free and conjugated IAA accumulations in the cutting bases were observed in the IAA treated cuttings, but that did not promote the percentage of cuttings rooted. IBA gave the best propagation results, as 80% of cuttings formed roots during the first month of rooting. This indicates the influence of IBA on early root development, independently of IAA (Štefančič et al. 2007). Similarly, it was also reported for *Malus* microcuttings. IBA induced more roots than IAA although it was converted to IAA only at very low levels, suggesting that either IBA itself was active or that it modulated the activity of IAA (Van Der Kriken et al. 1992). Many other investigations have shown that IBA (indole-3-butyric acid) has a greater ability to promote adventitious root formation in comparison to IAA (Spethmann and Hamzah, 1988; Riov, 1993; De Klerk et al., 1999; Ludwig-Müller, 2000). It is more stable and less sensitive to the auxin degrading enzymes (Nordström et al. 1991; Epstein and Ludwig-Müller, 1993; Riov 1993). IAA is rapidly metabolized by the peroxidase, acting as an IAA-oxidase, with the strongest activity during the root initiation phase (Caboni et al. 1997, Nag et al. 2001). IAA is partially protected against oxidation when transformed into one of its conjugated forms, from amongst which IAAsp (indole-3-acetylaspartic acid) is the more abundant in various plant species (Norcini and Heuser 1988, Nordström et al. 1991). Rapid conjugation also prevents the overaccumulation of IAA within the tissue, which is a more common occurrence in exogenous auxin applications (Faivre-Rampant et al. 2002). When hydrolyzed, IAAsp can serve as a slowly released source of free auxin during the advanced stages of the rooting process (Epstein and Ludwig-Müller 1993; Riov 1993). IBA conjugates are an even better source of free auxin, because they are more resistant to the in vivo degradation, whilst IAAsp is subjected to oxidation into biologically inactive products (Riov 1993).

Several other positive physiological effects of IBA treatment have been recognised over recent years. It has been demonstrated that exogenous IBA, when applied to the rooting zone, activates the sugar metabolism that releases energy and provides carbon skeletons for the synthesis of other essential compounds such as proteins (Agullo-Anton et al. 2011). Furthermore, IBA is reported to increase the rate of ethylene biosynthesis, and an auxin-ethylene relation during root development has been shown by the number of isolated mutants that have resistance to both hormones (Sachs 2005, Werner et al. 2008, Perilli et al. 2010, Pop et al. 2011).

Several authors reported that the stimulation of ARF in cuttings by exogenous indole-3-acetic acid coincided with increased sugar availability at the site of root primordial development. It was discovered that exogenous auxin applied to the rooting zone stimulates the mobilization of carbohydrates in leaves and the upper stem and increases the translocation of assimilates towards the rooting zone in order to release energy, and provides carbon skeletons for the syntheses of other essential compounds such as proteins (Haissig 1989). Auxin application to the stem base of carnation cuttings raised sugar levels within the same tissues during rooting and counteracted the transient sugar depletion in leaves (Agullo-Anton et al. 2011).

SEAWEEDS ARE AN IMPORTANT SOURCE OF HORMONES

Seaweeds are an important source of plant growth regulators, together with organic osmolites, amino acids, mineral nutrients, vitamins, and vitamin precursors. Over recent years, the use of natural seaweed as a plant growth stimulant has allowed for its substitution in place of conventional synthetic growth regulators and fertilizers (Sahoo 2000, Khan et al. 2009). In particular, Kelpak® [Kelpak® Kelp Products (Pty) Ltd] contains 11 mg/L IBA, and 0.031 mg /L kinetin, as well as a high amount of amino acids, vitamins, and mineral nutrients. This biostimulant is prepared by a cell burst process from brown algae *Ecklonia maxima (*Osbecki) Papenfuss, located in the cold waters along the Atlantic coast of Southern Africa. Recent reports have shown that low levels of *Ecklonia maxima* concentrate, when applied as a foliar spray or root drench, increases seedlings' qualities and survival, improves root-growth, vegetative and reproductive growth, flowering, fruit production, and the yields of many crop plants and vegetables (Crouch and van Staden 1993, 1994; van Staden et al. 1994; Kowalski et al. 1999; Arthur et al. 2003; Khan et al. 2009). Whilst there is a growing acceptance that seaweed liquid fertilizers (SLF) are tools when addressing the need for improved current crop production practices for long-term sustainability, the margin for beneficial action regarding Kelpak® during the vegetative propagation of ornamental cuttings, was found to be rather narrow. Urbanek Krajnc et al. (2012) performed a time-course analysis of the photosynthetic pigment, phenolic, and protein contents within the leaf tissues of *Pelargonium peltatum* 'Ville de Paris Red' from the initial cuttings to market maturity, in regard to brown algae [*Ecklonia maxima (*Osbeck) Papenfuss] extract treatment (Kelpak®). A positive impact by the 0.5% and 1% Kelpak® treatment on the protein accumulation within the leaves of pelargoniums was monitored. Elevated protein contents within the Kelpak®treated samples could be attributed to a high concentration of IBA within the Kelpak® extract, since it is well-known that IBA has a positive-effect on those enzymes involved in nitrogen assimilation (Kaur et al. 2002, Hayat et al. 2009). Beside the exogenous IBA, kinetin, and other seaweed components, especially nitrogen, amino acids, and vitamins, may have a synergistic impact on protein accumulation within Kelpak®treated cuttings. The results are in accordance with other seaweed treatment experiments. For example, Beckett et al. (1994) indicated increased levels of nitrogen within the leaves of nutrient-stressed tepary beans after treatment with Kelpak®. Furthermore, increased

total protein values were also reported in *Arachys hypogaea* by Sridhar and Rengasamy (2010) after treatment with brown algae *Sargassum wightii* extract.

Kelpak® treatment was also found to significantly affect the concentration of phenolic compounds within the pelargonium leaves (Urbanek Krajnc et al. 2012), thus the involvement of IBA in phenylpropanoid pathways was hypothesized. Similarly, Fan et al. (2011) reported that root treatment with brown algae (*Ascophyllum nodosum*) extract elicits the phenylpropanoid and flavonoid pathways in spinach, but the mechanism remains unexplained.

In our previous study (Urbanek Krajnc et al. 2012), we also demonstrated that Kelpak® treatment of *Pelargonium peltatum* Ville de Paris Red' cuttings can maintain the functionally active green leaves due to increased photosynthetic pigment accumulation, thus improving the 'whole plant' functional integrity following the insertions of cuttings into the soil. Maintaining the functionally active green leaves during root induction is crucial, since root formation relies on an adequate supply of carbohydrates from the source leaves to the region of root regeneration (Druege and Kadner 2008). Carbohydrate depletion is compensated for by current photosynthesis (Haissig 1989, Rapaka et al. 2005, Ahkami et al. 2009, Agullo-Anton et al. 2011). However, the excised cuttings of most plant species, including control pelargonium, show low photosynthetic rates, a decrease in chlorophyll contents and an increase in β-carotene and lutein unless first roots are formed, which could be explained by lower water potential due to severance of cuttings (Druege and Kadner 2008, Urbanek Krajnc et al. 2012). In contrast, Kelpak® treated pelargonium cuttings showed higher pigment concentrations when compared to the controls, reflecting a readjustment of the photosynthetic carbon metabolism. Urbanek Krajnc et al. (2012) assumed that the presence of IBA in the Kelpak® extract may increase chlorophyll concentration in leaves of cuttings. It was reported previously that IBA treatments cause an enhancement of chlorophyll contents within different stem cuttings (Mustafa 1996, Kaur et al. 2002). Furthermore, seaweed products are known to enhance plant chlorophyll content and reduce leaf senescence as a result of a reduction in chlorophyll degradation, which might be caused in part by betaines within the seaweed extract (Khan et al. 2009). It has been reported recently that SLF prepared from different brown algae species has a beneficial effect on photosynthetic pigments' contents. The application of SLF derived from the brown algae *Ascophyllum nodosum* in combination with standard fertilization, increased the leaf chlorophyll content, photosynthesis, and transpiration rates in tomato, cucumber, dwarf French beans, wheat, barley,maize, apples, grapes, and strawberries (Blunden 1991, Whapham et al. 1993, Blunden et al. 1997, Spinelli et al. 2010). Thirumaran et al. (2009) reported that, a SLF prepared from the brown algae *Rosenvingea intricata*, increased the total chlorophyll and carotenoids in *Cyamopsis tetragonoloba*. Furthermore, Sridhar and Rengasamy (2010) reported that groundnut treated with *Sargassum wightii* extract showed enhanced concentrations of photosynthetic pigments in leaves.

Nethertheless, the biostimulatory effect of Kelpak® treatment on primary and secondary metabolism of pelargonium cuttings was reflected in significantly increased root fresh mass, shoot/root ratio, and maximum number of leaves (Urbanek Krajnc et al. 2012). To sum up, higher photosynthetic pigment concentrations in cuttings treated with auxin-containg SLF, significantly contribute to increased photosynthetic activity and enhanced production of photoassimilates. Consequently, higher sugar levels during rooting are positively correlated with less expressed leaf senescence, higher survival rate, increased root formation and more efficient nutrients' uptake of cuttings.

CONCLUSIONS

In the presented review, causal interactions between auxins and specific primary and secondary metabolites were analysed during the process of ARF. It has been shown how the applications of different auxin sources or compounds inhibiting basal auxin transport modulate different metabolites during ARF. Taken together, exogenous auxin application promotes ARF in several ways. It has a direct influence on root induction at the stem bases of cuttings, as well as a stimulatory effect on sink activity and the translocation of assimilates towards the stem base. Auxin further inhibits leaf senescence, increases the chlorophyll contents and the photosynthesis in leaves, and accounts for the plasticity of shoot/root relations.

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Circadian behavioral patterns and body weight affect ammonia emissions in a pig fattening room

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ABSTRACT

The aim of this study was to analyze the effects of the circadian behavioral patterns and body weight of pigs on the ammonia emissions produced in a pig fattening room. The microclimatic conditions, ammonia concentration and carbon dioxide were measured with a special monitoring device. The behavior of pigs (laying areas and urination places) was observed indirectly. The ammonia emissions had a linear relationship with the urination frequency on the solid floor and the body weight (BW) of pigs. In the group with a body weight of 25 kg (BW25), the urination frequency on the solid floor increased by 1% as the ammonia volatilization increased by 0.1 g pig-1 day-1. These levels were doubled in the BW50 group and trebled in the BW75 group. The pigs also had a circadian behavioral pattern. The urination frequencies on the solid floor start to increase in the morning (06–12 h) and with the highest in the afternoon (12–18 h) when the pigs were more active. This resulted in the immediate higher production of ammonia emissions. The pig BW did not affect the laying preferences of the pigs.

Key words: ammonia emissions, behavior, body weight, pig

INTRODUCTION

Pigs prefer different places for laying and dunging (Stolba and Wood-Gush, 1989; Ekkel et al. 2003). To meet the preferences of pigs with respect to the performance of the laying area, the floor type, pen partition, and local climate (temperature, air velocity, and concentration of aerial pollutants) should be taken into consideration. It is well known that pigs prefer to lay on a solid floor (Aarnink et al. 1997). The laying area should have the correct climate conditions so the pig is in its thermal comfort zone. Therefore, it is important to ensure the appropriate air flow over solid floor. This can be achieved by placing a closed partition beside the solid floor (Hacker et al. 1994, Bjerg et al. 2000), which also prevents possible attacks from other pigs (in neighboring pens), and/or by controlling the ventilation rate and temperature (Saha et al. 2011). After the thermal neutral zone is provided, pigs prefer to lay on the warm, solid floor. Baxter (1982) also found a close relationship between the location of the feeder and the laying area, and concluded that pigs tended to lay near the feeder more frequently, while they avoided excreting in that area. This behavior is observed particularly when the feeders are situated in the corners of the laying area (Wiegand et al. 1994).

The preferences of pigs for dunging areas have also been analyzed. Several studies have confirmed that pigs prefer to defecate away from their laying area (Steiger et al. 1979, Stolba and Wood-Gush 1989). In general, pigs excrete on a slatted floor (Aarnink et al. 1997). It has also been suggested that pigs prefer to excrete more frequently in well-illuminated areas (Randall et al. 1983, Taylor et al. 2006). However, after pigs have defined their places for laying and dunging, they usually do not change their behavior (Hacker et al. 1994), unless the conditions are modified. Several studies have confirmed the impact of increased temperatures on the defecation behavior of pigs. At higher ambient temperatures (25ºC for 25 kg pigs and at 21ºC for 80 kg pigs), pigs search for a cooler place to lay (Steiger et al. 1979, Fraser 1985, Huynh et al. 2005, Aarnink et al. 2006). At the same time, pigs increase their rate of urination on the solid floor (Huynh et al. 2005, Aarnink et al. 2006). This behavior is undesirable, mainly because of higher ammonia emissions (Aarnink et al. 1996).

However, very little is known about changes in pig behavior (laying and excreting) after their daily activities are modified during the different fattening stages. This knowledge could help us to understand how ammonia emissions are related to pig behavior on a daily basis. Therefore, the aim of the present study was to analyze the behaviors of pigs (urination and laying) and to determine their relationship to ammonia emissions at different pig body weights (BW; 25 kg, 50 kg, and 75 kg).

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MATERIALS AND METHODS

Animals and feed

This study was performed in accordance with Slovenian legislation related to animal protection (Animal Law 2007). Nine replicate treatments of commercial pigs (Landrace x Large White dams and Landrace x Pietrain sires) were used and each comprised 11 pigs. Three replicates represented one of three testing groups: BW = 25 kg (BW25), BW50, and BW75. All of the groups were provided with pelleted commercial feed, which is normally used in Slovenia (metabolic energy = 12 MJ kg^{-1}; crude protein = 169 g kg^{-1}; lysine = 10 g kg^{-1}). Feed was provided around 7:00 h in the morning and 15:00 h in the afternoon, while drinking water was available ad libitum. No health problems were observed during the study.

Housing

The study was conducted in the Pig Research Center at the Faculty of Agriculture and Life Sciences (University of Maribor, Slovenia), where a naturally ventilated system was used. Fresh air entered the room through a diffuse ceiling and there was one exhaust unit with a mechanical ventilator. In the present study, the same pens were used for all test groups (Fig. 1). Each pen had a solid floor (75%) and a slatted floor (25% slatted; Fig. 1). The feeder was positioned in the laying area (solid floor) while the drinking bowl was placed on the opposite side at 34 cm above the slatted floor. The pen partition was closed on both sides of the pen. The slurry pit was 2.0 m deep and was cleaned 2 weeks before the start of the research. The floor pens were cleaned once each day (in the morning).

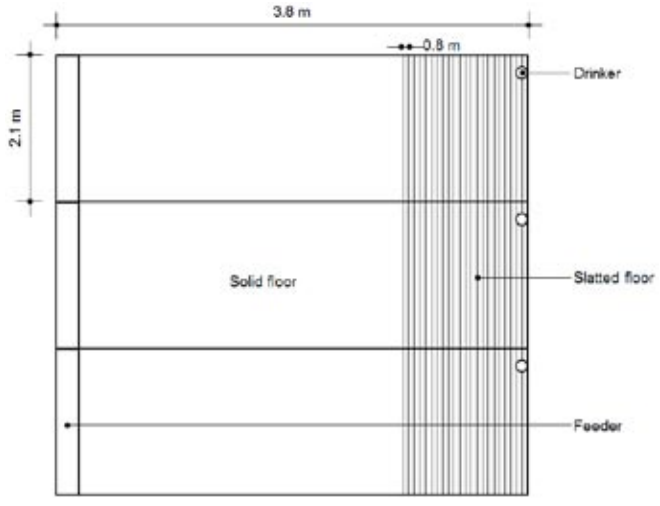

Figure 1: Layout of the pens used for each group

Measurements and calculations

Production parameters

All pigs were weighed individually at the beginning and at the end the experiment. The amount of feed per day was regularly checked. The feed intake was recorded per replicate and it was later divided by the number of pigs to calculate the daily intake per pig.

Behavioral parameters

The pigs were recorded with video cameras (The Imaging Source GigE color camera, 1024 × 768 pixels, Germany). Inox video cameras were placed on the wall. Recordings were made using one camera per indoor pen area. The behavior of the pigs was videotaped continuously. The videos were saved directly onto a Thecus N5200b server (USA) and analyzed subsequently using the GeoVision program (USA). A low level of human activity was preferred during recording (for control). The following were recorded from the videos.

1. Continuous analysis of pig urination. The analysis distinguished urination on the solid floor and on the slatted floor.
2. Scan sampling was performed every 1 h to determine the laying positions pigs, i.e., solid or slatted floor. Pigs laying on the border between the solid and slatted floors were assigned to the floor type where most of their body mass resided.

Microclimatic parameters

An Echo monitoring system (Echo d.o.o., Slovenia) was used to measure microclimatic parameters. The following parameters were measured.

- Indoor/outdoor temperature, air velocity, and relative humidity
- Ammonia concentrations and carbon dioxide

Indoor/outdoor temperature, air velocity, and relative humidity

The temperature was measured with resistance thermometers (Pt 100), which had a range of –40°C to +60°C and an accuracy of 1°C. The relative humidity was determined using a semiconductor sensor with an accuracy of ± 1% and the air velocity was measured using a thermal thin-layered detector. All of the data (temperature, relative humidity, and air velocity) were recorded four times each day and were stored automatically on an internal hard drive.

Ammonia concentration and carbon dioxide

The ammonia concentration was measured indoors using an electrochemical galvanic cell, where each group was tested separately. The sensor measurement accuracy was 1 ppm and the measurement range was 0–100 ppm. The ammonia concentration was not measured in the incoming air. In general, the incoming ammonia concentration was <5% of the ammonia concentration in the exhaust air (Aarnink et al. 1995). Therefore, the measured ammonia concentration could differ by up to 5% from the actual concentration.

Carbon dioxide was measured using two-dimensional infrared (IR) spectrum detectors. Sensors were used to determine the concentration of carbon dioxide indoors, where an absorption frequency of 4.265 μm was used. The measurement range for carbon dioxide was 0–2000 ppm,

with a measurement accuracy of 1 ppm.

All of the sensors were calibrated manually each week. During calibration, the ammonia sensor was set to 0 ppm and the carbon dioxide sensor, was set to 385 ppm.

Data analysis

The ammonia emissions were calculated from the ammonia concentrations recorded during the research. The total heat production rate of the fattening pigs was calculated first, according to the equation provided by CIGR (2002):

$$\Phi_{tot} = 5.09\, m^{0.75} + [1 \times (0.47 + 0.003\, m)]\, [n \times 5.09\, m^{0.75} \quad 5.09\, m^{0.75}] \qquad \text{(Eq. 1)}$$

where:
Φ_{tot} - Total animal heat dissipation in animal houses, W
m - Body mass of the pigs, kg
n - Daily feed energy relative to φ_m
Φ_m - Pig maintenance (5.09 $m^{0.75}$), W

The carbon dioxide production was calculated based on the total heat production data, according to the following CIGR method (2002):

$$c = \frac{0.185 \times \Phi_{tot}}{1000} \times 24 \qquad \text{(Eq. 2)}$$

where:
c - CO_2 production, m^3 day^{-1}
Φ_{tot} - Total animal heat dissipation in the animal houses, W

The ventilation rate was calculated using equation 3 (carbon dioxide balance), according to De Sousa and Pedersen (2004):

$$V = \left(\frac{c}{CO_2 \text{ in} - CO_2 \text{ out} \times 10^{-6}} \right) \qquad \text{(Eq. 3)}$$

where:
c - CO_2 production, m^3 day^{-1}
CO_2 in - CO_2 concentration measured indoors, ppm
CO_2 out - CO_2 concentration measured outdoors (385 ppm), ppm

The ammonia emissions were calculated using equation 4, according to CIGR (2002) and De Souza and Pedersen (2004), as follows:

$$AE = V \times AC \times [(17/22.4) \times (273.13/(273.13 + t)] \qquad \text{(Eq. 4)}$$

where:
AE - Ammonia emission, g day^{-1}
V - Ventilation rate, m^3 day^{-1}
AC - Ammonia concentration, ppm
t = Temperature, ºC

The ammonia emissions and behaviors of the pigs (laying and urination) were calculated on a daily basis, as well as separately in the morning (06–12 h), afternoon (12–18 h), night (18–24 h), and early in the morning (00–06 h) to determine the circadian variation.

All of the data were analyzed using IMB SPSS 20. The production parameters (BW, mean daily gain, and mean feed intake) were calculated using the descriptive statistics and expressed as the mean ± SE for each separate test group (BW25, BW50, and BW75). The circadian behavioral patterns in the urination frequency and laying area preferences were compared for groups BW25, BW50, and BW75 using the Chi-square test. The data were expressed as the mean ± SD. The statistical differences are shown in the Figures as *** ($P < 0.001$).

RESULTS AND DISCUSSION

Production parameters

The pigs in the BW25, BW50, and BW75 groups had mean BWs of 22.0 kg (SE = 0.66), 48.8 kg (SE = 0.29), and 76.2 (SE = 0.78) kg; mean daily gains of 656 g d^{-1} (SE = 8.9), 742 g d^{-1} (SE = 1.6), and 638 g d^{-1} (SE = 1.7); and mean feed intakes of 1.48, 2.28, and 2.30, kg d^{-1}, respectively.

Behavioral parameters

Behavioral patterns

Figure 2 shows the differences in the urination frequencies on the solid floor for the different test groups (BW25, BW50, and BW75). Pigs in the BW25, BW50, and BW75 groups had daily urination frequencies of 3.2, 2.7, and 3.9 times per pig, respectively, and there were no significant differences in the urination frequencies (Fig. 2) between replicates within the testing groups ($P = 0.690$). The urination frequencies were affected significantly by the BW during the overall fattening period (between BW25, BW50, and BW75) ($P = 0.000$). A comparison of BW25 and BW50 (BW increase of 55%) showed that the urination frequency on solid floor increased by 10% (48 ± 5% vs. 58 ± 5%). As the BW increased further by 35% (group BW50 vs. group BW75), the urination frequency on the solid floor increased by an additional 16% (74 ± 5%). Previous studies did not document an increased urination frequency on solid floors. As the BWs of the pigs increased, they urinated more often on the solid floor because there was insufficient space per pig (Aarnink et al. 1996, Spoolder et al. 2000, Savary et al. 2009). It has also been observed that pigs change their laying behavior. With increasing body weight, pigs lay more often on slatted floors (Aarnink et al. 1996, Savary et al. 2009). However, this was not the case in the present study.

Figure 3 shows an example of the laying preferences of pigs on the solid floor. As the BW increased, the laying pattern of pigs on the solid floor remained relatively constant. In BW25, BW50, and BW75, the pigs had laid on the solid floor for averages of 80%, 80%, and 76% of the time, respectively, but only 6%, 7%, and 7% of the time on the slatted floor. High differences in the preferences for laying areas were observed within the groups. For groups BW25, BW50, and BW75, the

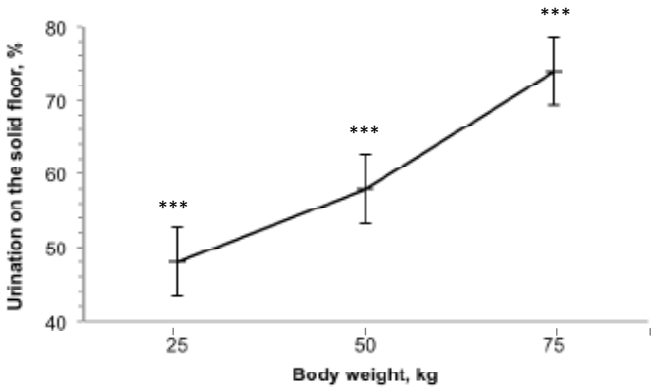

Figure 2: Urination pattern on the slatted floor with increasing pig body weight

SDs were 18%, 17%, and 25% for pigs laying on the solid floor, whereas the SDs were 5%, 5%, and 9% for pigs laying on the slatted floor (Fig. 3).

Circadian behavioral variation

Table 1 shows the frequency of urination and laying by fattening pigs in the different groups (BW25, BW50, and BW75) at different times (00–06 h, 06–12 h, 12–18 h, 18–24 h). In general, pigs urinated at least in the night time (18–24 h) and early in the morning (00–06 h). In the morning (06–12 h), the urination frequency increased (P = 0.000), with a peak in the afternoon (12–18 h) (P = 0.000) in all groups. At the

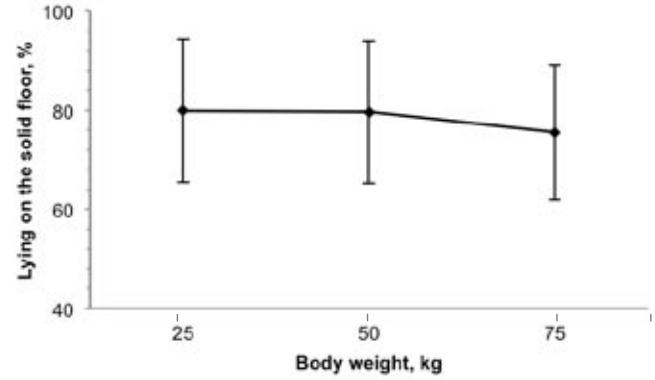

Figure 3: Laying pattern on the solid floor with increasing pig BW

same time as the peak urination frequency on the solid floor (in the afternoon at 12–18 h), the pigs laid less on the solid floor (P = 0.000), regardless of their BW. In a previous study, there was a relationship between laying on the solid floor and excretion behavior. Huynh et al. (2005) and Aarnink et al. (2006) found that pigs excreted more frequently on the solid floor and that they lay less on the solid because of increased laying on the slatted floor. However, this change in behavior was observed when the ambient temperature increased. This was not the case in the present study (conducted at thermal neutral ambient indoor temperatures). We found that the pigs did not change their behavior and they laid on the slatted floor more. In the afternoon, the pigs became more active (or laid less) (P = 0.000). Previous studies have shown that pigs start to urinate when they increase their activities, such as feeding (Groenestein et al., 2003, de Sousa and Pedersen 2004, Guarino et al. 2008). Guarino et al. (2008) observed that most pigs urinated 1 h or 2 h after feeding. Urination may also be related to the lighting regime because it has been reported that pigs prefer to urinate more often when light is provided (Randall et al. 1983, Taylor et al. 2006), which is probably because pigs are more active during the day time (Olsen et al. 2001). In the present study, artificial indoor lighting was provided for 8:00 h, starting at 7:00 h in the morning, and feed was provided at the same time (7:00 h and 15:00 h).

However, it is not known why the pigs change their laying behavior and/or excreting behavior in the afternoon (12–18 h). It is possible that the pigs are more active during feeding time and with a higher light intensity. Thus, they start to urinate more often on the solid floor (near the feeder).

After comparing the different groups (BW25, BW50, and BW75), we found that the BW75 pigs urinated significantly less often (P = 0.000) in the afternoon (12–18 h) and more (P = 0.000) in the night time (18–24 h) compared with early in the morning (00–06 h) (P = 0.000) relative to BW25 and BW50 pigs. We hypothesized that pigs had to change their urinating patterns as their BW increased, which normally leads to space limitations. Weary el al. (2008) found that pigs with a lower status in the social hierarchy avoid contacts at the feeder and drinker. Therefore, it is possible that pigs with a low status are more active and urinate more often during the night time because they avoid contacts at the feeder and the drinking bowl. It is also possible explanation that the pigs with a higher BW (BW75) were more thirsty and hungry in the night time.

Ammonia emissions

Figure 4 shows the relationship between the urination frequency (%) on the solid floor and the ammonia emissions (g pig^{-1} day^{-1}). The regression, line Y = 0.671X – 22.795 (R^2 = 94%), shows that the ammonia emission increased linearly with increasing urination on the solid floor. The regression analysis detected a clear effect of the frequency of urinating on the solid floor on the ammonia emissions (P = 0.000). This relationship between the urination frequency and ammonia emissions has been reported in previous studies. Urine puddles on the solid floor are important sources of ammonia emissions, which are produced due to urea degradation by the enzyme urease (Groenestein et al. 2007; Ivanova-Peneva et al. 2008). Therefore, ammonia volatilization from the urine pools is related to the proportion of the solid floor in the pen. Hoeksma et al. (1992) observed that ammonia volatilization was about 30% of the total ammonia emission in pens with 62% slatted floor. Aarnink et al. (1996) also analyzed ammonia volatilization in pens with 25% and 50% slatted floors. With a 50% slatted floor, the ammonia emissions from the solid floor contributed 23% of the total emissions, and 40% of the emissions when a 25% slatted floor where used. These data shows that as the proportion of slatted floor is reduced, the ammonia emissions increase from urine pools on the solid floor, while there is a simultaneous decrease in the proportion of ammonia volatilization produced from the slurry pit. In the present study, we also calculated the impact on the am-

Table 1: Frequency (mean ± SD) of urination and laying patterns of pigs in the different bodyweight groups (BW; 25 kg, 50 kg, and 75 kg) and at different times (00–06 h, 06–12 h, 12–18 h, and 18–24 h)

Variable	BW, kg	Time, h			
		06–12	12–18	18–24	00–06
Total urination, % of total urination frequency	25	27 (1)	39 (2)	19 (1)	16 (2)
	50	21 (2)	49 (3)	14 (1)	14 (4)
	75	20 (1)	29 (2)	26 (1)	25 (1)
Urination on slatted floor, % of total urination frequency	25	9 (2)	23 (2)	11 (2)	6 (1)
	50	7 (3)	21 (3)	4 (2)	9 (3)
	75	8 (1)	8 (1)	7 (4)	2 (1)
Urination on solid floor, % of total urination frequency	25	18 (1)	18 (2)	7 (3)	9 (2)
	50	12 (3)	23 (5)	6 (2)	7 (2)
	75	32 (4)	25 (1)	16 (3)	25 (1)
Total pigs laying, % of total pigs laying	25	93 (1)	64 (2)	88 (1)	95 (1)
	50	90 (2)	60 (3)	89 (2)	91 (1)
	75	88 (2)	45 (3)	94 (1)	88 (1)
Pigs laying on solid floor, % of total pigs laying	25	88 (5)	58 (4)	80 (5)	92 (4)
	50	87 (4)	53 (3)	80 (6)	80 (4)
	75	88 (5)	35 (3)	75 (5)	88 (5)

monia emissions of the urination frequency on the solid floor by different pig groups (BW25, BW50, and BW75). When the urination frequency on the solid floor increased by 1%, the ammonia volatilization increased by 0.1 (SD = 0.01) g pig^{-1} day^{-1} in BW25, by 0.2 (SD = 0.02) g pig^{-1} day^{-1} in BW50, and by 0.3 (SD = 0.02) g pig^{-1} day^{-1} in BW75. Our results show that the ammonia emissions (due to the urination frequency on the solid floor) were also affected by the pig BW.

Figure 5 shows the relationship between BW (BW25, BW50, and BW75) and ammonia emissions (g pig^{-1} day^{-1}). The linear regression line (Y = 1.6x + 4.4 (R^2 = 90%)) shows that the ammonia emissions increased from approximately 9.5 g pig^{-1} for the BW25 pigs to approximately 25.3 g pig^{-1} for the BW75 pigs. The regression analysis showed that the ammonia emissions increased significantly with pig BW (P = 0.000).

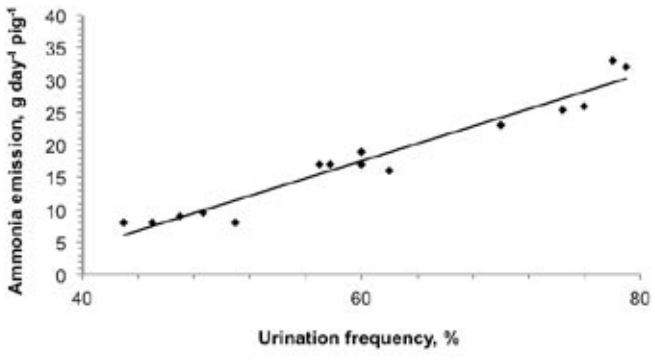

Figure 4: Relationship between the urination frequency (%) and ammonia emissions (g pig^{-1})

Circadian variation in ammonia emissions

Figure 6 shows the relationships between the ammonia emissions (g pig^{-1}) and urination frequency (%) for different pig BW groups, and at different times (in the morning (06–12 h), in the afternoon (12–18 h), in the night (18–24 h), and early in the morning (00–06 h)). The peak ammonia emissions coincided with the peak urination frequency on the solid floor. The linear regressions showed that 86%, 65%,

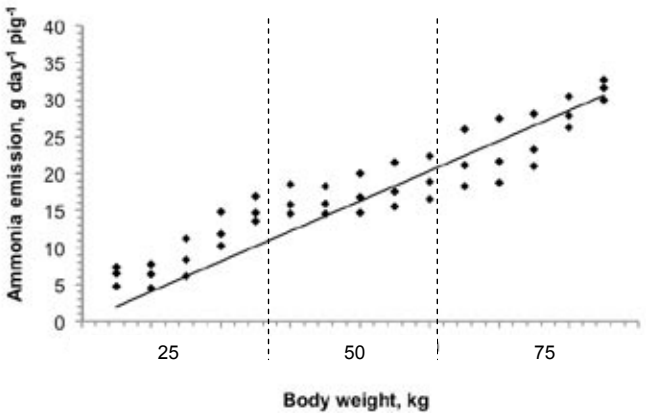

Figure 5: Relationship between pig body weight (BW25, BW50, and BW75) and ammonia emissions (g day^{-1} pig^{-1})

and 77% of the variation in ammonia emissions by groups BW25, BW50, and BW75, respectively, could be explained by the urination frequency. The data shows that there was no time gap between the moment of urination on the solid floor and the increase in the ammonia emissions. These findings agree with Elzing and Swierstra (1993) who found that the hydrolysis of urea in urine to ammoniacal N occurs immediately when urine splashes on the floor.

Therefore, it is not surprising that the lowest ammonia emissions were recorded in the night (18–24 h) and early in the morning (24–06 h), because pigs urinated the least (Table 2) at these times in all three groups. The ammonia emissions increased significantly (P = 0.000) in the morning (06–12 h) in all groups (BW25, BW50, and BW75). In BW50 and BW75, the emissions increased by 16% and 18%, respectively, while in BW25, the ammonia emissions increased at twice that rate (36%) and increased by a further 7% in the afternoon (12–18 h). However, the ammonia emissions increased by 10% in BW50 but only by 3% in BW75. Thus, the differences between the highest ammonia emissions (in the afternoon) and the lowest (early in the morning) declined as the pig BWs increased. The difference between the afternoon and early in the morning for BW25 was 36%, while for BW50 it was 25%, and 21% for BW75.

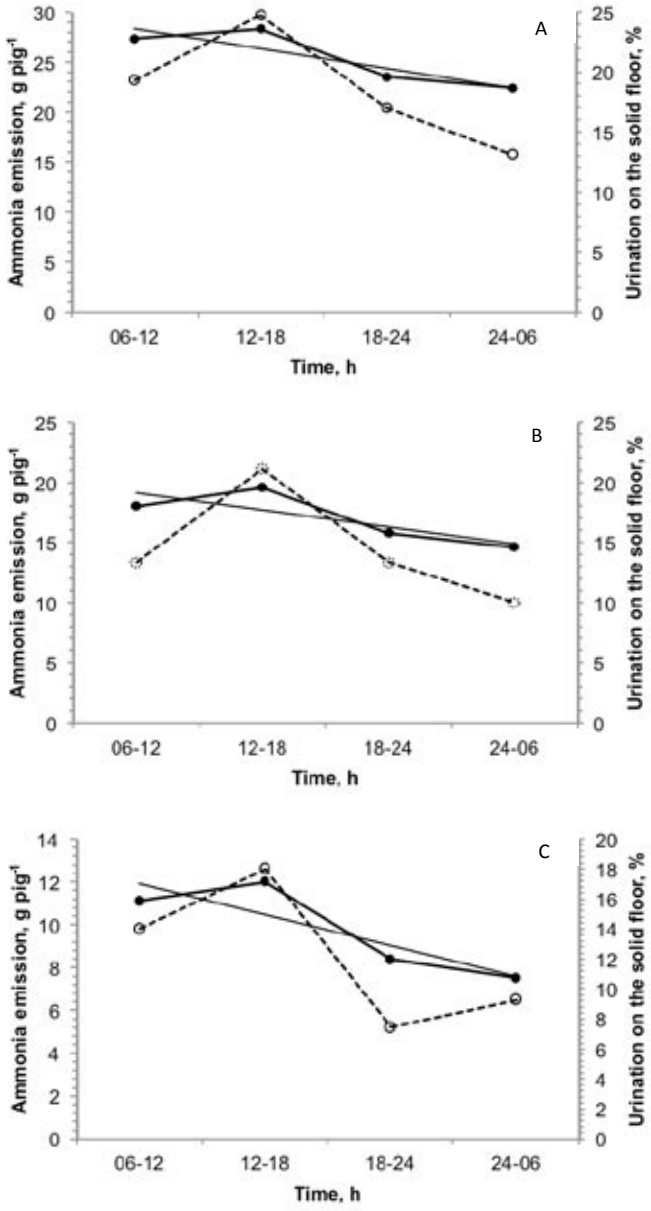

Figure 6: Relationship between ammonia emissions (g pig^{-1}) (—) and the urination frequency (%) (- - -) by different pig BW groups (C) BW25, (B) BW50, and (A) BW75

CONCLUSIONS

This study of the circadian behavioral patterns and changes in the BW of pigs detected a clear effect on the ammonia emissions produced in the pig fattening room. As the pig BW increased from 25 kg to 75 kg, the daily urination frequency on the solid floor increased by 26%, but there were no differences in the laying preferences. After analyzing the circadian variation in the urination frequency, we found that the urination frequency started to increase in the morning (06–12 h), probably due to the lighting regime and feeding time, with the highest peak in the afternoon (12–18 h) in all BW groups (BW25, BW50, and BW75). The ammonia emissions had a linear relationship with the urination frequency on the solid floor which differ among the pig BW groups. Our results show that the ammonia emissions produced in pig fattening room started to increase in the morning and the highest emissions were observed in the afternoon. These results suggest that the standard pen cleaning regime (in the morning) could be adjusted to the fouling of the pens and to production of ammonia emissions. Therefore, we recommend that the floor pens should be cleaned in the afternoon when the ammonia emissions are highest.

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Genetic diversity of Christmas rose (*Helleborus niger* L.) natural local populations as revealed by AFLP markers

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ABSTRACT

Three naturally-growing local populations of Christmas rose (*Helleborusniger L.*) from two locations in Slovenia and one from neighbouring Croatia, were selected for molecular analysis based on AFLPs. 27 primer pairs were evaluated during the selective amplification step of the AFLP, and ten primer combinations were generated on the basis of the number of bands. The results from molecular analysis showed that the variation within populations was larger than that between populations. The molecular analysis showed that the genetic differences amongst populations were not high enough to state that the individuals of *H. niger* were grouped in clearly distinctive clusters. Small differences between the two Alpine populations (Bohinjska Bela and Peca) suggest that these are not isolated at the level of sexual reproduction. The migration of genes through pollen dissemination is probably always present, although pollen cannot migrate far.

Key words: Christmas rose, *Helleborus niger*, population diversity, molecular markers, AFLP

INTRODUCTION

The Christmas rose (*Helleborusniger L.*) that belongs to the family Ranunculaceae (Tamura 1993) is becoming more and more popular on the market as an ornamental plant (Armstrong 2002). However, it is a relatively new species regarding market-oriented production. It is important for its promotion to organise efficient and systematic genetic breeding, and to improve production technology. The efficiency of genetic breeding depends on several factors, such as available genetic resources, breeding methodology, breeders' experiences, and financial support.

Slovenia, one of the smaller European countries, appears to be very rich in terms of its biotic diversity (Martinčič, 1999). The main reasons for such diversity are its specific geographical position as well as its diverse climate, relief, and bedrock. *H.niger* grows within all phytogeographical regions (Wraber 1969) (Fig. 1) and Slovenia is considered as being at the centre of diversity regarding this species. The variation within the genus Helleborus appears to be extremely high and the majority of the more important traits are at least partly influenced by the environment (Šušek et al. 2005). Reliable data about existing diversity and variation within germplasm collections will be extremely important for breeders in the future.

The traditional evaluation of genetic variability, based on a description of morphological traits, cannot always ensure accurate estimates of the genetic variation. Many more reliable estimates of genetic variation can be obtained by using biochemical (e.g. isozymes) and molecular markers.

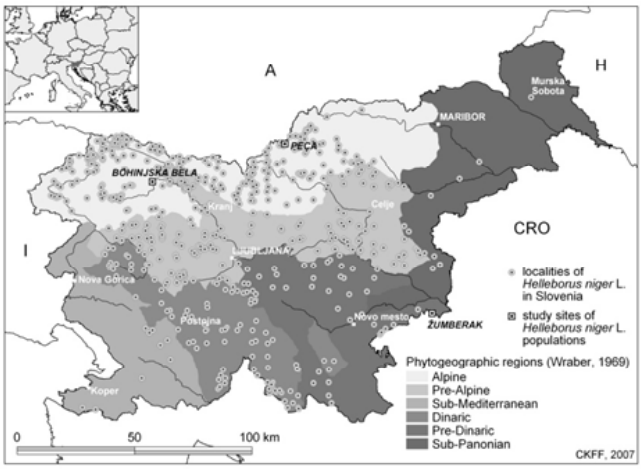

Figure 1: Localities of *Helleborusniger* in Slovenia (Jogan 2001). The map was adapted with the permission of the CKFF (the Slovenian Centre for the Cartography of Fauna and Flora)

The AFLP technique has been successfully used for evaluating genetic relationships within the genus *Helleborus*. Meiners et al. (2011) analysed the taxonomic subdivision within the genus *Helleborus*. The results supported the previously-suggested division of the genus and thereby approved AFLP data as being applicable for phenetic

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analyses. AFLP markers have found the widest application regarding the analyses of genetic diversity below the species level within plants, particularly during investigations into population structure and when differentiating between both cultivated and natural/rare populations (Gupta et al. 1999, Nissim et al. 2004). Such analyses are crucial from the applied breeding point of view and for genetic conservation, and the rapidity with which AFLP markers enable the delivering of crucial information within intense time-constraints.

Patzak (2001) compared its potential for assessing the genetic diversity of ten hop (*Humulus lupulus* L.) varieties by using RAPD, STS, ISSR, and the AFLP molecular approaches. All the molecular methods accurately distinguish between the tested varieties, with the exception of three clones, which can only be distinguished by AFLPs. The similarities between varieties were revealed by using cluster analysis, and similar results were found for all the molecular methods.

When AFLPs reveal low-levels of genotypical differentiation between sub-populations' and relatively high variations within populations, they cannot be used for determining the populations' geographical origins. The reason could be the migration (of seeds or pollen) amongst sub-populations. This migration is probably present frequently. If seed migrations were excluded, the most important factor could be the migration of pollen. Examples can be the studies of wild North American populations of the weedy-plant *Arabidopsis thaliana* (L.) Heynh, as conducted by Jorgensen and Mauricio (2004), and the analysis of population structure regarding *Arctophila fulva* var. *pendulina* (Laest) Holmb. (Kreivi et al. 2005). Similar results were also obtained by Nissim et al. (2004) when studying the genetic variations of wild populations and cultivated genotypes of *Anemone coronaria* (L.). Most of the genetic variations (about 80 %) were documented within populations and only about 20 % between populations.

Data about the level of genetic diversity within and between populations of *H. niger* are crucial for genetic breeders. They are also very important for the establishment and management of germplasm collections. Information about the genetic structuring of populations is crucial during evalution studies. However, at this moment in time, no such information is available.

AFLPs can be very helpful for studying the genetic relationships amongst populations, and for determining or identifying ecotypes, hybrid lines, and cultivars. The AFLP technique can also represent a useful tool during the breeding process of the Christmas rose, thus enabling early determinations of traits that can only normally be determined when the plants are fully mature, and traits that can be determined only within specific environments (e.g., in the presence of certain diseases or pests). We studied the genetic structures of three naturally-grown populations that were chosen on the basis of results from morphological analysis. The aim of this study was to estimate the usefulness of the AFLP molecular technique when examining the genetic diversities amongst and within populations.

MATERIALS AND METHODS

Plant material

The molecular analysis included randomly-chosen Christmas rose genotypes (individual plants) from two locations within Slovenia (Bohinjska Bela and Peca) and one in neighbouring Croatia (Žumberak). These locations were selected according to the results of morphological analysis that involved 8 populations (Šušek et al. 2005). From each selected population, we took (for the molecular analyses) 16 plants (a total of 48 plants). Each individual within the population was collected at a distance of 50-500 m from the others, in order to avoid clones.

DNA isolation and AFLP analysis

Genomic DNA was extracted from the leaves according to the established CTAB method using NucleoSpin Plant kits (Macherey-Nagel, GmbH & Co.KG, Düren, Germany) by following the manufacturer's protocol. The DNA quantities and qualities were estimated by measuring the absorbance at 260 nm and 280 nm, using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany), and by gel electrophoresis by means of 0.8 % agarose gel electrophoresis.

AFLP analysis was performed according to Vos et al. (1995), with slight modifications. The AFLP core reagent and starter primer kits were purchased from ScienceTec (LI-COR Inc., Biotechnology division, Lincoln, Nebraska, USA). Approximately 100 ng of genomic DNA was double-digested using the restrictive enzymes mix *Eco*RI/*Mse*I. The genomic DNA was incubated within a 5 × reactive buffer (50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, and 250 mM K-acetate). Digestion was carried out within a final volume of 12.5 µl at 37 °C for 2 h, and then heated to 70 °C for 15 minutes in order to deactivate the enzymes.

*Mse*I and *Eco*RI adapters (Table 1) were subsequently ligated to the digested DNA fragments by adding 12 µl of adapter ligation solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate), and 0.5 µl of T4 DNA ligase (1 unit/µl in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50 % glycerol (v/v)). The ligation was incubated for 2 h at 20 °C. After the reaction, the digested-ligated DNA fragments were diluted (1:10) and used as templates for the first amplification reaction (the pre-amplification step prior to the selective amplification).

Pre-amplification reactions were performed within a 25.5 µl volume containing a 2.5 µl of 10 × PCR buffer for AFLP (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl), 20 µl of pre-amplification primer mix (Table 2), 2.5 µl of diluted ligation and 0.5 µl of Taq DNA polymerase (Roche Molecular biochemicals, Penzberg, Germany). The PCR amplification was carried out in a PTC-100 Programmable Thermal Controller using the following cyclic profile:

Table 1: Adapter and +3 primer sequences (5'-3') used for AFLP analysis

Name	Enzyme	Type	Sequence (5'-3')
E-0	*Eco*RI	Adapter	5'-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'
M-0	*Mse*I	Adapter	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-5'
M-CAG	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACA G
M-CTG	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACT G
M-CAC	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACA C
M-CTA	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACT A
E-ACA	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAC A
E-ACC	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAC C
E-AAG	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAA G
E-ACT	*Eco*RI	IRDye 800 labelled primer +3	GAC TGC GTA CCA ATT CAC T
E-AGC	*Eco*RI	IRDye 800 labelled primer +3	GAC TGC GTA CCA ATT CAG C

Step	Temperature (°C)	Time	
1. denaturation	94	30 seconds	20 cycles total
2. annealing	56	60 seconds	
3. extension	72	60 seconds	
4.	4	hold	

After the reaction, pre-amplified DNA fragments were diluted (1:40) and used as templates for the selective amplification reaction. A volume of 11 µl PCR reaction combined with a 2 µl diluted (1:40) pre-amplification product, 0.5 µl of IRDye 700 labelled *Eco*RI primerA, 0.5 µl of IRDye 800 labelled *Eco*RI primerB, 2 µl of *Mse*I primer, and 6 µl of TaqMix. The TaqMix for 33 reactions contained 40 µl of 10×PCR amplification buffer (100 mM Tris-HCl, pH 9, 15 mM $MgCl_2$, 500 mM KCl), 158 µl of deionised water and 2 µl Taq DNA polymerase (5units/µl) (Roche Molecular Biochemicals, Penzberg, Germany). The second amplification was carried out in a PTC-100 Programmable Thermal Controller by programming a touch-down cyclical profile (Don et al., 1991). Touchdown PCR was performed by decreasing the annealing temperature from 66 °C to 56 °C by 0.7/30 s increments per cycle with each of the initial 12 cycles, followed by a touchdown annealing temperature for the remaining 25 cycles at 56 °C for 30 s with a final cycle of 72 °C for 1 min and a hold at 4 °C.

Following the amplification reaction, each product was mixed with 5.0 µl of blue-stop solution (98 % formamide,10 mM EDTA pH 8.0, and bromo-phenol-blue and xylene cyanol as tracking dyes). The resulting mixtures were heated at 94 °C for 3 min., and then quickly cooled by ice and covered to reduce exposure to light. The samples were stored at -20 °C for long-term usage

Gel electrophoresis was conducted using a Li-Cor Long ReadIR DNA sequencer (LI-COR Inc., Biotechnology division, Lincoln, Neb., USA).

Data analysis

Image analysis was performed using Saga 2 software (LI-COR Inc., Biotechnology division, Lincoln, Neb., USA). After the automated analysis, the placements of lanes, calibrations, desmile lines, and the scoring of bands was reviewed by the visual inspections of two independent readers. Only the bands' scores by both readers were accepted as reproducible. When considering of bands, the diffused or faint bands were not taken into account and neither were those bands that occurred at the extremes of the amplified size-range. Infrequent bands were eliminated. Only the bands with a frequency above 20 % were taken into account. Each AFLP marker was treated as a unit character and scored as a binary code (1/0).

Only polymorphic bands were used during the construction of a binary matrix, reflecting the presence or absence of those fragments obtained by AFLP, in different genotypes. The estimates of genetic similarities (GS) amongst all genotypes were calculated according to the Jaccard similarity coefficient (1908). The genetic distance was calculated as GD = 1 – J, using the data from the Jaccard's similarity coefficient matrix. The resulting distance matrices were subject to clustering methods by the Neighbour Joining Tree (NJTree) method.

The reliabilities and robustness of the phenograms were tested by bootstrap/jackknife analyses by drawing 1,000 samples.

Data analyses were done by Darwin, the non-commercial

software of CIRAD (Perrier et al. 2003). A bootstrap value was given to each edge and indicated the occurrence frequencies of this edge in the bootstrapped trees.

Principal component analysis (PCA) was used in an attempt to detect geographical patterns amongst the *H.niger* populations.

RESULTS

During the selective amplification step, the duplex of one *Mse*I with two IRDye labelled *Eco*RI primers was used for exploiting the capability of the LI-COR system when detecting two different dyes. The 27 primer pair combinations were evaluated with regard to their capacities for generating polymorphic bands using 4 genotypes, randomly chosen from the 3 populations being investigated (Table 2).

According to their capacities to generate polymorphic bands, it was decided to use a five duplex of one *Mse*I with two IRDye labelled *Eco*RI primers: (1) M-CAG, E-ACA (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (2) M-CTG, E-ACC (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (3) M-CAC, E-AAG (IRDye 700 labelled), E-ACT (IRDye 800 labelled); (4) M-CTA, E-ACC (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (5) M-CTG, E-ACA (IRDye 700 labelled), and E-ACT (IRDye 800 labelled). Each selected primer combination generated a greater number of bands. The number of polymorphic fragments for each primer pair varied from 5 (for M-CTG/E-ACC) to 23 (for M-CAG/E-ACA), with an average of 8.9 per primer pair (Table 3).

The sizes of the AFLP fragments were determined by comparing an AFLP standard marker to the AFLP patterns. The AFLP fragments' sizes ranged from approximately 45 to 400 base pairs (bp). The polymorphic fragments were distributed across the entire size-range, with the major proportion being between 75 and 300 bp.

In order to assess the usefulness of AFLPs as phenetic markers, a similarity-matrix based on Jaccard's coefficient was constructed for estimating the level of relatedness amongst individuals from the three natural populations used during the study. Calculation of the Jaccard coefficient was based on the presence or absence of discrete characters (AFLP markers). The similarity matrix was then used to cluster the data using the NJTree method and to conduct PCA analysis. The 48 individuals appeared as being scattered along the resulting dendrogram constructed by the Jaccard coefficient, and by the NJTree clustering method (Fig. 2). Individuals from the Žumberak population were clustered into two sub-clusters of 5 and 10 individuals, and a sub-cluster of 6 individuals was identified for the Peca population. All the other individuals were clustered in groups of two to four individuals, irrespective of their geographic origins. The resulting dendrogram showed low bootstrap values for the main edges with a certain number of null values.

The similarity matrix was also used as input data for principal component analysis (PCA). The scatter-plot representation of the PCA showed a clear separation of 9 individuals from the Žumberak population in relation to the first two principal axes of variation (Fig. 3). When the plan was formed by axes 1 and 2 only, 18.11 % (9.76 % + 8.35 %) of the total variation was explained by the markers. The second

Table 3: Number of polymorphic fragments and their size-range

Primer combination	Scored polymorphic bands	Fragment size-range (bp)
M-CAG/E-ACA	23	50-370
M-CAG/E-AGC	10	50-370
M-CTG/E-ACC	5	40-340
M-CTG/E-AGC	6	50-360
M-CAC/E-AAG	13	45-340
M-CAC/E-ACT	5	50-400
M-CTA/E-ACC	6	50-370
M-CTA/E-AGC	8	45-370
M-CTG/E-ACA	8	50-255
M-CTG/E-ACT	5	50-370
Totals	89	-
Mean	8.9	-
Number of primer combination	10	-
Maximum	23	-
Minimum	5	-
Standard deviation	5.59	-

Table 2: Primer pair combinations tested for conducting selective amplification

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC		○		●	○		●	
E-AAG	○	●						
E-ACA		○	●			●		●
E-ACC				●	●	○	●	○
E-ACG	●	○		○				
E-ACT	○	●				○		○
E-AGC			●				●	
E-AGG		○	●		○			

plane (axes 3 and 4) accounted for 13.29 % (6.82 % + 6.47 %) of the total variation. This meant that the markers could not explain the highest portion of variation amongst the studied individuals. Similarly, in the case of the third plane (axes 4 and 5), only 12.17 % (6.47 % + 5.70 %) of the total variation could be explained.

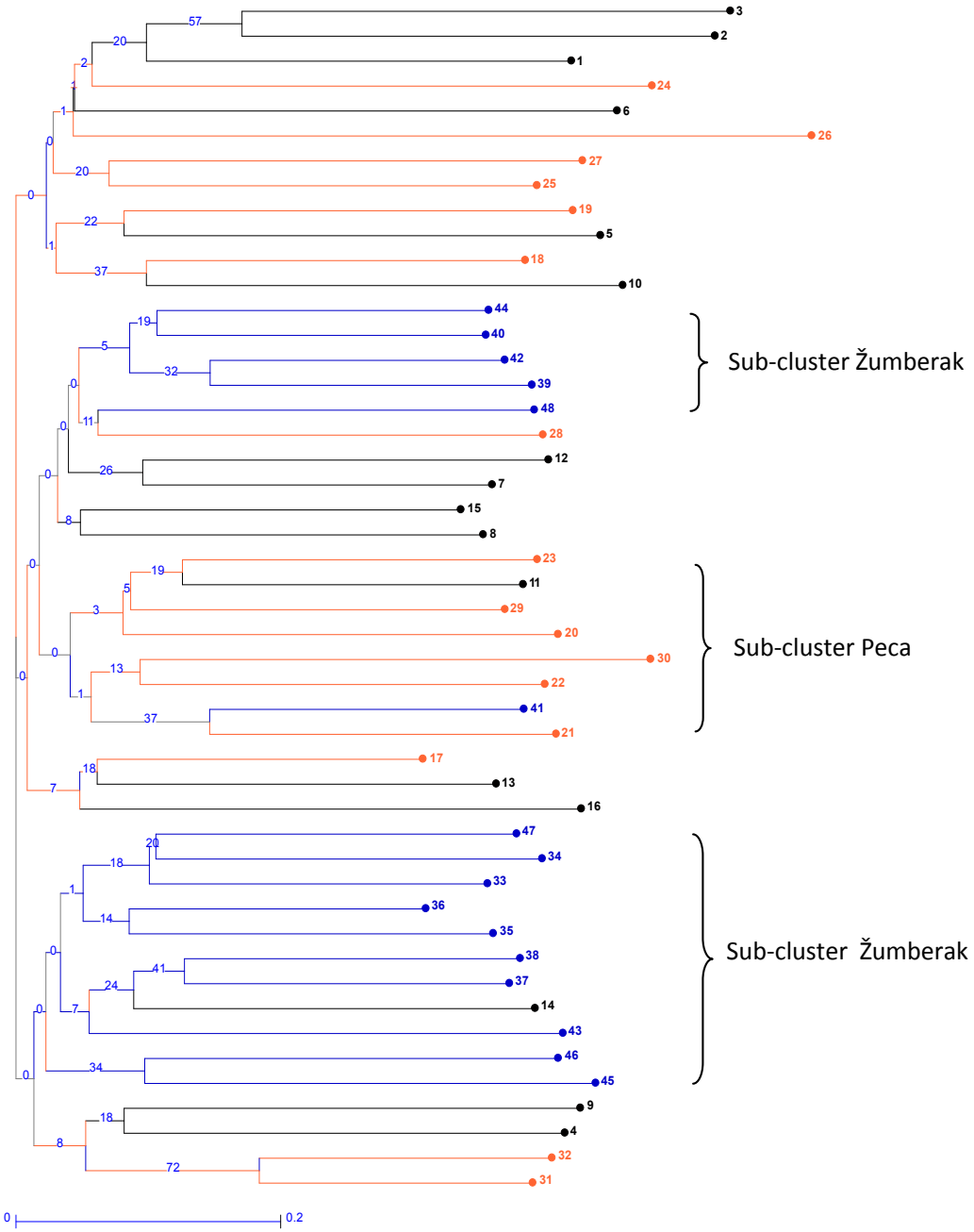

Figure 2: Unrooted dendrogram of individual plants from wild populations based on individual scoring for each AFLP band. The numbers represent individuals from various populations: Bohinjska bela (from 1 to 16); Peca (from 17-32); Žumberak (from 33 to 48). Neighbour-joining clustering was performed, using the genetic similarity coefficient of Jaccard calculated between every pair of the genotype. Bootstrap values (%) for each branch point are indicated

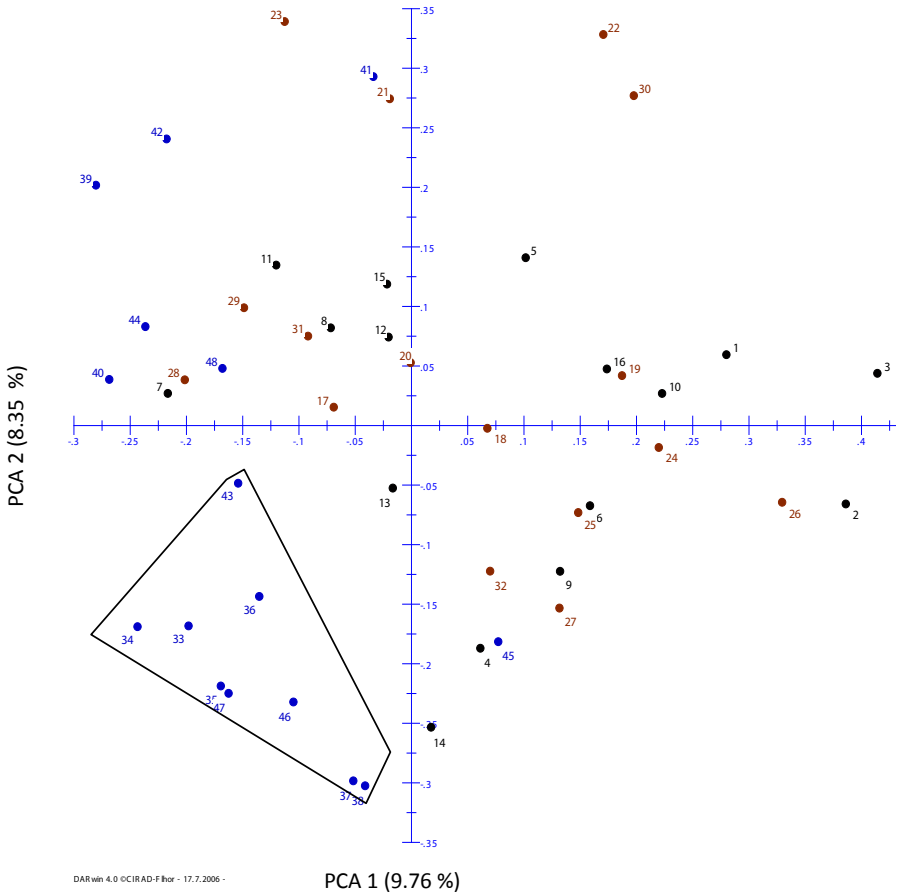

Figure 3: Principal component analysis (PCA) of AFLP data for *H. niger* naturally-growing populations. The numbers represent individuals from various populations: Bohinjska Bela (from 1 to 16); Peca (from 17-32); Žumberak (from 33 to 48). The percentage of total variance explained by the first two principal components is shown on the abscissa and ordinate axes, respectively.

DISCUSSION

The AFLP technique was used for evaluating the genetic diversities within and between three naturally-grown populations of Christmas rose. As this was the first work on the natural diversity of *H.niger*, sixteen individuals were selected from each population in order to gain some knowledge of their genetic diversities. For technical reasons, we decided to use different individuals as repetitions, being aware that this could not eliminate all the possible errors associated with the representativeness of the data.

For the AFLP assay, we used a relatively wide-range of primer combinations. From amongst the 27 AFLP primer combinations tested, 17 were discarded owing to the reduced number of obtained fragments, polymorphic or not. The genetic diversities were finally evaluated using 10 primer combinations that generated 89 polymorphic fragments and made the fingerprinting of each individual genotype possible.

Evaluation of the genetic diversity of the Christmas rose within naturally-occurring Slovenian populations did not show strong differentiations. Although several individuals from the same location appeared to be very tightly clustered, many of them were distributed all over the dendrogram (Fig. 2). The heterogeneity within clusters was higher than between clusters. PCA analysis showed that only a low percentage of the total variations amongst these individuals could be explained by the markers (Fig. 3). However, geographical distance tended to be a factor associated with genetic similarities. The Žumberak population, which was geographically the most distant when compared to the Bohinjska Bela and Peca populations, also tended to be genetically different to the two latter populations, to a certain extent (Figs. 2 and 3). A similar situation was observed by Šušek et al. (2005) on the basis of morphological traits where, on the basis of the first principal component, the Žumberak population was separated from the Peca and Bohinska Bela, which could not be distinguished one from the other.

One interpretation of the structure in the neighbour joining-tree and in the scatter plot from the PCA analysis could be that these Alpine populations were not genetically isolated. The only substance that enables genetic communication amongst populations is probably pollen, which is carried by various insects (e.g., bees).

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Body morphological characteristics of honey bees

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ABSTRACT

Honey bees (*Apis mellifera*) consist of more than 24 different subspecies. Most of these subspecies have been classified according to their morphological characteristics, and morphological characteristics thus have an important role in the classification aspects of honey bees. Different sets of wing and body morphological characteristics have been used to characterize and classify the subspecies by many authors and for various reasons. These characteristics were defined over time and combined from various studies. Wing venation characteristics have been studied more intensely than other body morphological characteristics. Up to now there are no specific review articles focus mainly on body morphological characteristics. Therefore, the available information about sampling method, measuring method, importance and factors affecting these characteristics were reviewed to present essential conclusion and recommendations for researchers.

Key words: *Apis mellifera*, morphometry, body characteristics, honey bees

INTRODUCTION

The honey bee, *Apis mellifera* L., is globally widespread with a wide diversity of subspecies. These subspecies can be classified with morphometric tools (Ruttner et al. 1978). Many studies have been undertaken on honey bees using morphological characteristics (e.g. Abou-Shaara et al. 2012 and Garnery et al. 2004). These characteristics can be divided into three major groups; which are length measurements, color measurements, and wing venation characteristics. Wing venation characteristics were previously reviewed intensively by Abou-Shaara (2013). Here, the major studies that have been done using body morphological characteristics were reviewed to provide recommendations about sampling, measuring method and limitations of body morphological characteristics.

IMPORTANCE OF MORPHOLOGICAL MEASUREMENTS

Body morphological characteristics can be measured for different reasons. A major use is to characterize honey bee races and individuals (Ruttner 1988, Meixner et al. 2007), but also to determine the degree of hybridization with foreign races (Radloff et al. 2003 and Bienefeld et al. 1996). Also, for the discrimination between honey bee subspecies (e.g. Abou-Shaara and Al-Ghamdi 2012, Tofilski 2004). Moreover, morphological characteristics were measured to investigate the impacts of imported queens on honey bee populations (Guler 2010) or to check populations purity (Miladenovic et al. 2011). Multiple body characteristics, including wing length, wing width and tongue length were used to differentiate between honey bee subspecies (Buco et al. 1987, Rinderer et al. 1993, Crewe et al. 1994, Ftayeh et al. 1994, Diniz-Filho and Malaspina 1995, Szymula et al. 2010).

Tongue length was found to be an indicator of geographical variation in some studies (Marghitas et al. 2008, Morimoto 1968, Souza et al. 2002). Proboscis length was also found to be the most differentiated characteristics between *A.m.mellifera*, *A.m.carnica* and *A.m.caucasica* (Szymula et al. 2010). In addition, body measurements may show correlations to honey yield. Kolmes and Sam (1991) found that honey production was highly correlated to overall size, corbicular area and wing measurements in Carniolan honey bees. Body characteristics may thus be used for indirect prediction of colony productivity or for selection of productivity where honey bees with bigger legs and wings have higher power flight and could gather more pollen and nectar for brood rearing and consequently colony population (Mostajeran et al. 2006). There is a positive correlation between honey production and corbicular area (Milne and Pries 1984). Szabo and Lefkovich (1988) found that honey production had significant and positive correlations with both fore and hind wing area. Mostajeran et al. (2002) found that honey production was related to tongue length, fore wing length and width, hind wing length, leg length, femur length, tibia

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length and metatarsus width. Waddington (1989) found a correlation between body size and colony productivity. Edriss et al. (2002) indicated that honey production can be improved through selection of the forewing width. Therefore, there is evidence that body morphological characteristics are very important and correlated with colony productive characteristics. However, it must be noted that these relations may attribute to specific conditions rather than indicating general rules.

COLLECTION OF BEE SAMPLES

At least 15 honey bee workers should be collected from each colony during the morphological analysis (Ruttner et al. 2000, Sheppard and Meixner 2003, Meixner et al. 2007, Guler 2010) and at least eight colonies per district can be considered sufficient for morphological study (Abou-Shaara et al. 2012). However, Miguel et al. (2011) used only one honey bee worker per colony for geometric morphometric but generally more is required to obtain reliable results. Samples can be collected in a number of ways (i) directly from brood comb according to Padilla et al. (1992). (ii) shaking bees into a jar, (iii) collecting forager bees (iv) taking one-day old bees by placing sealed brood combs into incubators. Collected bees can be preserved in 95% ethanol until dissection (Arias et al. 2006) or in 70% ethanol (Adl et al. 2007) or killing by a deep-freezer and then dissected (Abou-Shaara et al. 2012). Also, the temporary preparation of the samples can be used (Miladenovic et al. 2011).

It needs to be taken into account that there are some factors that can impact on the morphological characteristics. Comb cell size has an impact on morphological characteristics (Ruttner 1988, McMullan and Brown 2006, Gencer and Firati 2005) where workers emerged from large wax cells have larger morphological characteristics. Sample size and time of taking the samples thus could affect comparisons between different data for body morphological characteristics. In general, it is very important to take samples for morphological analysis at the same time for all studied replicates and try taking samples from new combs and under the same condition of feeding as possible.

METHODS OF MEASURING BODY MORPHOLOGICAL CHARACTERISTICS

After the collection of the samples, samples can be mounted on sticky pieces as described by Abou-Shaara and Al-Ghamdi (2013) to facility characteristics measuring. Also, other mounting methods for body parts (e.g. double glass slides) can be used (Abou-Shaara et al. 2011). Several methods have been used to take the body measurements; (i) stereomicroscope with an ocular micrometer (Ruttner et al. 1978, Mattu and Vermam 1984, Edriss et al. 2002, Souza et al. 2002, Sirali et al. 2003, Gencer and Firati 2005, Cakmak et al. 2006, Tan et al. 2006, Mostajeran et al. 2006, Adl et al. 2007, Marghitas et al. 2008). (ii) Photomicroscope (Morris-Olson 2002). (iii) Projecting mounted slides onto a TV screen

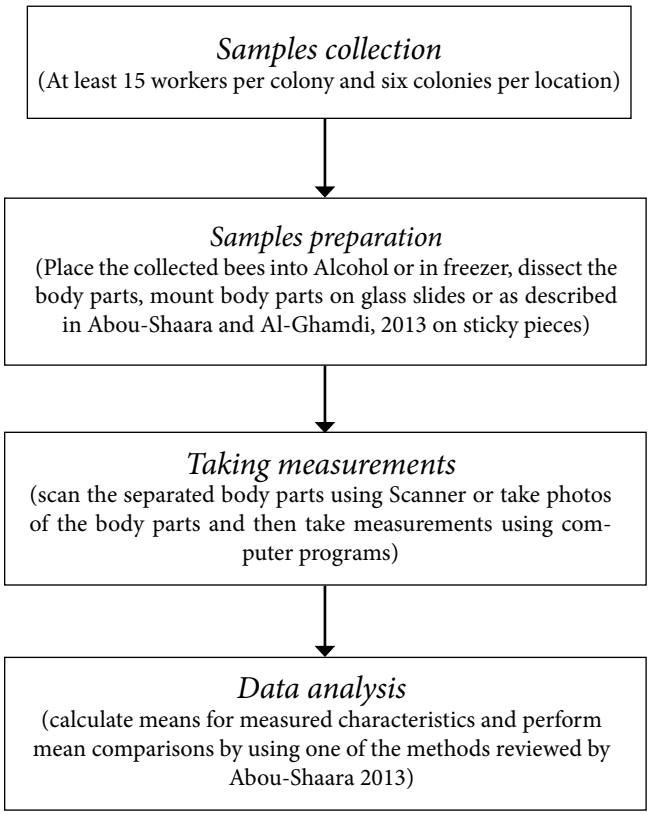

Figure 1: The suggested steps for the morphometric analysis based on body characteristics

(Kandemire et al. 2000). (iv) Using computer-based methods mainly for forewing and some body characteristics (Meixner 1992, Rinderer et al. 1993, Ruttner et al. 2000, May-Itza et al. 2001, Kamel et al. 2003, Schnider et al. 2003, Sheppard and Meixner 2003, Haddad and Fuchs 2004, Meixner et al. 2007, Shaibi et al. 2009, Miladenovic et al. 2011, Abou-Shaara et al. 2012, Abou-Shaara and Al-Ghamdi 2012). (v) Special programs using image analysis systems and the IMAGO program (Padilla et al. 1992). Computer program Object-Image Pre2.11, at a scale of 150:1 (Jones et al. 2005). The image analyzer IMAGEPRO plus version 3.0.1 for Windows 3.1 and Media Cybernetics were also used Andere et al. (2008).

Moreover, Abou-Shaara et al. (2011) presented a simple method for measuring body and wing morphological characteristics by using photoshop program; this method was called Scan Photo method while Miladenovic et al. (2011) used AutoCAD program to take the measurements. In general, all the previous methods can be used but it is preferable to use computer-based method to save time where microscopic methods were found to be time-consuming (Szymula et al. 2010). Moreover, any suitable software can be used in combination with camera or scanner to take the measurements (Fig. 1).

BODY CHARACTERISTICS

Various body characteristics of honey bees were measured by many authors (e.g. Meixner et al. 2007, Shaibi et al. 2009, Miladenovic et al. 2011, Abou-Shaara et al. 2012, Abou-Shaara

and Al-Ghamdi 2012). Commonly, body characteristics are normally measured according to Ruttner et al. (1978) and Ruttner (1988). These characteristics can be divided according to body parts into; head, thorax and abdomen characteristics. (i) head characteristics include; head capsule width (HCW) and length (HCL), antenna length (AL) and number of segments (ANS), compound eyes length (CEL) and width (CEW), and tongue length (TonL). Some authors also studied the mandible length (ML) beside some other characteristics. (ii) thorax characteristics; fore wing length (FWL) and width (FWW), hind wing length (HWL) and width (HWW), number of hooks (NH), thorax width (ThW), femur length (FL), tibia length (TL), basetarsus length (BL) and width (BW), and pollen basket size (PBS), brush hair rows number (HN). (iii) abdomen characteristics; lengths of tergit 3 (T3) and 4 (T4), body size (T3+T4), length of hairs on tergit 5 (HLT5), pigmentation of tergit 2-4, length of sternite 3 (LS3), wax mirror length (WML) and transversal (WMT) and sting shaft length (StL). In general, measurements have to be taken as the maximum distance and in units of millimeters (mm) except the number of hooks. Moreover, there are some indexes were revealed from these characteristics (e.g. forewing index = length /width of fore wing).

FACTORS AFFECTING BODY MEASUREMENT VARIATION

Previous works on honey bee workers showed that environmental factors have a major impact on morphological characteristics (Eischen et al. 1982, Milne and Pries 1984, Milne et al. 1986, Stanimirovic et al. 2008). Marghitas et al. (2008) found that in the mountain regions of Transylvania for example worker proboscis were longer 6.21 mm than that in lower regions 5.99 mm. The importation of honey bee subspecies into different areas might induce high levels of hybridization within populations (Garnery et al. 1998, Rortais et al. 2004, Alqarni et al. 2011) and produce subspecies admixtures (Arias et al. 2006). Also, migratory beekeeping may play a key role in forming differences (Marghitas et al. 2008). Morphological characteristics for uncontrolled honey bee populations showed low stability through time (Abou-Shaara et al. 2012). Thus to characterize uncontrolled populations, taking the characteristics mean for two successive years is highly recommended. Some other factors that may impact on wing and body morphological characteristics were reviewed by Abou-Shaara (2013).

CONCLUSIONS

Various methods were used in taking morphological measurements. However, computer-based methods using programs (e.g., Photoshop, image tool and AutoCAD) could be recommended to save time and obtain accurate measurements. It is worth noting that the sample size, sampling season, sampling technique and measuring method differ from author to another and from country to another and should be better harmonized. Therefore, it is recommended to use standard methods for measuring these characteristics to facility comparing results of different subspecies and countries. Figure 1 shows the recommended steps for the morphometric analysis based on body characteristics. Fifteen workers per colony and six colonies per district should be sufficient for sample size. Taking samples from colony combs are easier than forager bees. Ongoing evaluation of morphological characteristics could help in understanding racial fluctuations due to beekeeping, hybridization and environmental factors. In addition, morphological characteristics are also correlated with colony productive characteristics. As a result, body morphological characteristics can be used as a simple indicator for estimating fluctuations in genetic and productive characteristics of honey bee colonies. It is apparent that still more work is required to provide insights into the seasonal impacts on body morphological characteristics.

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The effect of seminal plasma alkaline phosphatase, fructose and aspartate-amino-transferase on non-return rate in bulls

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ABSTRACT

In this study some of biochemical constituens of Simmental bull (n=31) seminal plasma were deterimined using spectophotometric analysis for asparte-amino-transferase (AST), alanine-amino-transferase (ALT), alkaline phosphatase (ALP), adenosine-triphosphate (ATP), fructose (FRU) and Citrate (CIT). The fertility of bulls (n= 18) was measured by non-retun rate 90 after insemination (NR) with deep frozen semen in strows with 6×106 of spermatozoa . There was significant positive correlation between NR and ALP (0.725, P=0.00147) and significant negative correlation between NR and AST (-0.659, P= 0.00554) and FRU (-0.562, P= 0.0153), respectively. It is quite evident that inseminations using ejaculates with high activity of ALP (> 3530 U/L) results in better NR (69% vs. 65%, P=0.028). However, biochemical complexity od seminal plasma attemps to perform such simple correlations among seminal plasma components and NR are likely to produce inconsistent results.

Key words: bovine, bull, seminal plasma, alkaline phosphatase, non-return rate

INTRODUCTION

The bull semen is is combined secretion of testis, epididymis and the male accessory glands (prostate, seminal vesicles) wich form the bulk of the fluid portion of semen known as seminal plasma. The composition and reproductive functions of seminal plasma are well described in generaly (Henault et al. 1995, Maxwell et al. 1997, Vishwanath and Shannon 1997) and in detail by many authors (Killian et al. 1993, Ahmad et al. 1996, Henault and Killian 1996) especially concernig the role of plasma in artificial insemination proces (Töpfer-Petersen et al. 1998). Summarising all these research we could extract from present knowledge that seminal plasma provides a medium for motility of sperm after ejaculation and enhance fertilisation of the egg (Holt 1996).

In consonance with literature mentioned above, it would be likely that selected seminal plasma biochemical constituens - particularly enzymes - and some sugar that provide ATP through glycolysis play important role in sperm motility and fertility of the bull.

This study was undertaken to investigate the effect od particullarly biochemical constituens of seminal plasma on fertility in bull. We will show, in contrast to to widely accepted point of view, that impact on bull fertility is transmited only by very few inducers investigated here.

MATERIAL AND METHODS

The study was performed on 31 Simmental bulls aged 12 to 14 months individualy penned on AI station. The bulls were offered basal diet consisted of the mixture including 4 kg of hay, 20 kg of corn silage, 3 kg of concentrate feed and vitamin-mineral mix. The total daily nutriens was therefore 11.5 kg od dry matter, 112.6 MJ of metabolic energy and 698 g of crude protein.

Semen samples were obtained from bulls using an artificial vagina; from each bull two consecutive ejaculates were collected and pooled together in order to drain as possible the epidydimis and accessory glands. After this, pooled sample was splited in two portions: a portion to used immidiately for chemical analysis of ejaculate and an aliquot for artificial insemination.

Ejaculate specimens were centrifuged on 3000 g to separate seminal plasma from the spermatozoa.

Supernatant of the seminal plasma was analysed for asparte-amino-transferase (AST), alanine-amino-transferase (ALT), alkaline phosphatase (ALP), adenosine-triphosphate (ATP), fructose (FRU) and Citrate (CIT) using Boehringer commercial kits[3] on automatic photospectometric analyser.[4]

Because of low volume of seminal fluids from some

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ejaculates, certain investigations were unable to perform. So, the number of tests varies among the chemical analyses as shown in section of Results.

The semen freezing extender used in this experiment was egg yolk-citrate-glycerol with ethylen-di-amino-tetra-acetic acid. After cooling the semen was stored in 0.5 mL French strows with 6×106 of spermatozoa and immersed into the liquid nitrogen till insemination.

For artificial insemination we used randomly selected cows and heifers on the dairy farms in north-east Slovenian region. Non-return rate (NR%) of 18 bulls was recorded 90 days after insemination computing the field insemination records and expressed the the results as percentage of pregnant animals.

All collected data were analysed using descriptive statistic methods, correlation including partial correlation methods, and t-test for independent samples with algorithms from SPSS software[5]

RESULTS AND DISCUSSION

In Table 1 the results of descriptive statistics are summarized to present physiological concentration of biochemical constituens investigated here (except of NR90%). The reference values for yearling simmental bulls are not widely available in present literature. For example, Luthra et al. (1994) indicates the average concentration of 23.5 mmol/L of fructose for Simmental bulls 12-15 months of age wich is slightly higher than our results. In the same study they found significantly higher sperm concentration in the semen of bulls with lower concentration of fructose. Also, bulls with higher concentration of fructose had higher sprem motility. Fructose is syntesized in accessory gland of reproductive tract and spermatozoa convert fructose anaerobically to lactate wich leads, at least in bull, to consecutive high motility (Storey 2008).

[5] SPSS Statistics17.0 Command Syntax Reference 2008, SPSS Inc., Chicago, USA.

In Table 2, correlation analysis between fructose concentration and non-return data indicate significant negative relationship. This is consistent with idea of Lutra at. al. (1994) although we do not examined the sperm concentration. However, this relationship is partially influenced also by presence od AST in the seminal plasma. If we exclude AST from this calculaton correlation coefficient between FRU and NR intends to be lower t.i. -0.379 (P=0.163). Increase level of seminal fructose was allways accompanied with increase activity of AST (Table 2; r=0.44, P=0.167). The correlation coefficient between AST and NR was negative and significant (-0.659, P=0.00554) This suggest that failure in sperm cellular membrane after freezing and leakage of transaminases had detrimental effect of NR results, wich agree with findings of Dhami and Sahni (1993) for ALT (r=-0.736) but not for AST. Ejaculates analysed here with activity of AST above the median value (>126 U/L) have not significant lower NR (65% vs. 67% in below median value, P=0.222). In contrast, study of Herak et al. (1993) indicate some higher level of AST activity in bulls with lower NR (256 U/L vs. 218 U/L).

The role of ATP in sperm metabolism is well known (Minelli et al. 1995). The spermatozoa metabolize different substrate to obtain energy in the form of ATP. This is mainly achieved by exploiting the catabolic routes of the glycolytic Embden-Mayerhof pathway, wich is anaerobic and produces 2 moles of ATP per mole of glucose and the aerobical Krebs citric-acid cycle, wich produce 36 moles of ATP per mole of glucose (Jones and Murdoch 1996). Dephosphorylation of ATP is fundamental for activity of spermatozoa, so this relationship suppose to be correlated with their molitilty. ATP content in seminal plasma (Table 1) is much lower as found in the sperm cell (Söderquist 1991). This may be the reason that we did not find the correlation between ATP and NR% and agree with results of McLaughlin et al. (1994) for human spermatozoa.

The alkaline phosphatase in seminal lasma has two sources. The major part is prostatic and very smaller part comes from sprem cell itself. We found (Table 1) slightly less activity of ALP in seminal plasma as Pangawkar et al. (1988) 3653 U/L vs. 5100 U/L and 7100 U/L after freezing, respectively.

Table 1: Descriptie statistics of biochemical constituens in seminal plasma before freezing: asparte-amino-transferase (AST), alanine-amino-transferase (ALT), alkaline phosphatase (ALP), adenosine-triphosphate (ATP), fructose (FRU), citrate (CIT) and non-return rate 90 day after insemination (NR) of the same ejaculate

	AST U/L	ALP U/L	CIT mmol/L	ATP mmol/L	FRU mmol/l	ALT U/L	NR 90 day %
N	29	28	30	31	31	28	18
Mean	142	3653	4,33	7,48	19,65	5,0	66,13
Std. Error of Mean	18,6	392	,125	4,15	1,135	,76719	,740
Median	126	3530	4,4	76	19,800	4,5	65,25
Std. Deviation	100,43	2076,46543	0,686	23,10	6,319	4,06	3,142
Minimum	3,00	866,00	2,30	9,00	6,60	,00	60,30
Maximum	332,00	8055,00	5,220	133,00	29,00	20,00	71,20

Table 2: Correlation matrix among biochemical constituens in seminal plasma before freezing (asparte-amino-transferase AST, alanine-amino-transferase ALT, alkaline phosphatase ALP, adenosine-triphosphate ATP, fructose FRU and citrate CIT) of Simmental bulls and non-return rate 90 day after insemination (NR) of the same ejaculate. Values in cell are indicated as Pearson correlation coefficient (first row), two tailed probability (second row) and number of observations (third row)

	ALT U/L	ALP U/L	ATP mmol/L	FRU mmol/L	CIT mmol/L	NR 90 day %
AST	-0.153 0.436 28	-0.448 0.017 28	-0.104 0.592 29	0.441 0.017 29	0.179 0.352 29	-0.659 0.006 16
ALT	1	0.162 0.410 28	-0.0875 0.658 28	0.0365 0.854 28	0.0104 0.958 28	-0.358 0.899 15
ALP		1	0.129 0.506 29	-0.488 0.007 29	-0.180 0.350 29	0.725 0.001 15
ATP			1	0.174 0.350 31	-0.204 0.280 30	0.257 0.303 18
FRU				1	0.046 0.808 30	-0.562 0.015 18
CIT					1	-0.273 0.290 17
NR						1

Observed difference between fresh and post-thawed semen might reflect initial damage of the spermatozoan membrane during freezing, with subsequent increase in its permeability, resulting in leakage of ALP in the seminal plasma. The positive significant correlation of ALP vs. NR (0.562, P= 0.0153) shows the potential of prostate gland through their secretion on the biochemical enviroment necessary for oocyte fertilisation. There is no reliable information availible in the literature regarding the fertility of post-thawed semen and ALP activity in seminal plasma in the bull. For the buffalo (*Bubalus bubalis* v. Surti) found Dhami and Kodagali (1990) sperm post-thaw motility had significantly (P<0.01) negative correlations with the release of ALP (−0.673) from the sperm cell.

Using linear correlation coefficient, it was detected strong negative impact of AST activity and FRU concentration in seminal plasma on 90 day non-return rate; beside of this the positve impact of ALP activity was also measured.

However, ejaculates with activity of AST above the median value (>126 U/L) have not significant lower NR (65% vs. 67%, P=0,222) but ejaculates with above median concentration of fructose (>19,8 mmol/L) show slight tendency to lower NR (65% vs. 68%, P=0.065). It is quite evident that inseminations using ejaculates with high activity of ALP (> 3530 U/L) results in better NR (69% vs. 65%, P=0.028).

CONCLUSIONS

The presented results do not support the view that the sperm fertility, measured by Non-return method, could be seriously affected during the release of sperm cell enzymes into the fresh seminal plasma. Contrary to results for the cell enzymes, the plasmal concentration of mainly prostatic ALP indicated some value as an indicator of ejaculate fertility. Hence, only ALP before-freezing activity above 3653 U/L can be attributed to better fertility in the bull. To summerise from this study, biochemical complexity od seminal plasma attemps to perform such simple correlations among seminal plasma components and NR, are likely to produce inconsistent results and their role in the assessment of sperm function must therefore be called into question.

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Investigation of phytotoxicity regarding copper fungicides applied to apples

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ABSTRACT

The influence of adding kaolin clay (Cutisan), pine resin (Nu-Film), and milk proteins (ProNet-alfa) to copper fungicide spray at the same levels of phytotoxicity was tested regarding copper fungicides when applied to 'Golden Delicious' apples tested during a field-trial. Copper fungicides were applied 8 times per season using a standard orchard sprayer (250 l/ha of spray), starting at the BBCH 71 stage (fruitlets with more than 15 mm diameter); and continuing throughout the season until stage BBCH 79, at the rate of 250 g Cu^{++}/ha at each application. The phytotoxicity levels of traditional copper fungicides (sulphate, oxy-chloride, oxide, hydroxide) were compared to fungicides based on copper complexes (gluconate, EDTA chelate, peptidate and aminoacid complexes). Fruit disorders were evaluated (malformations, surface-russet). The level of reduction in phytotoxicity when adding kaolin clay was 10 to 80% and reached from 10 to 40% when adding pine resin. The addition of milk proteins in some fungicides reduced phytotoxicity by 5 to 10%, whereas in others a 15 to 30% increase was observed. The content of copper was determined in the fruits at harvest-time. The use of additives generally increased the copper residues of the fruits. The highest increase was determined for pine resin (5-100%), moderate for milk proteins (5-70%), and the lowest for kaolin clay (0-30%). The results of our trial showed that the tested additives had different effects on the reductions of phytotoxicity regarding copper products, and the intake of copper ions to apple fruits. They were very specific in terms of compatibility with different formulations of copper fungicides. In regard to compatibility, kaolin clay had the most neutral effect. As the addition of pine resin and milk proteins can significantly increase the copper content of fruits, they were less suitable as additives for the reduction of phytotoxicity within certain formulations of copper fungicides.

Kew words: apple, copper, phytotoxicity, additives, kaolin clay, pine resin, milk protein

INTRODUCTION

The application of copper fungicides is still one of the important options for controlling the fire-blight disease of apples caused by *Erwinia amylovora*. The most important period regarding fire-blight control is the blooming period (Clarke et al. 1993). In Slovenia, weather conditions during the apple blooming period are often unsuitable for infections from bacteria; therefore there are fewer cases of severe blossom-blight. On the other hand, there are several cases of late twig-blight that develops during the first part of June, or later when weather conditions are much more suitable for the development of bacteria. Inoculum sources are latently infected trees from which bacteria are dispersed to the young fast-growing twigs of adjacent trees.

During that period fruit producers have less chemical options for twig-blight control. Copper fungicides are one of the few. It is well-known that the application of copper fungicides during the period of fast fruit development causes a certain level of phytotoxicity which results in fruit-russet and malformations (Lešnik et al. 2011). Russet is a very complex physiological phenomenon that is caused by cuticle cracking due to a failure to keep up with the rapid growth of a fruit's internal tissues (Ashizawa et al. 2005). Russet usually starts in the early stages of a fruit's growth - shortly after full-bloom, corresponding to the period of greatest tangential growth (Noè and Eccher 1996, Peryea 2006). Russet leads to apple market value reductions because of the non-compliance with quality standards. Russeted fruits are normally downgraded when marketing. Despite a certain level of phytotoxicity, growers still apply copper products as they do not have other alternatives. The main alternatives under Slovenian conditions are phosetyl-Al-based fungicides and *Bacullus subtilis* products. Fruit producers seek solutions on how to reduce the phytotoxicities of copper fungicides. According to the information given by pesticide manufacturers the phytotoxicities of copper sprays can be reduced to a certain level by using special additives.

The aim of our research was to test the levels of phytotoxicity regarding different copper fungicides when applied several

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times during a season to 'Golden Delicious' apples, and to check the possibility of reducing the levels of phytotoxicity by adding additives based on kaolin clay, milk proteins, and conifer resin. The tested additives are promoted by industry as suitable for organic fruit production systems because they are produced from natural substances.

MATERIALS AND METHODS

Experimental orchard and trial design

The field-trial was carried out at the experimental station of the Faculty of Agriculture and Life Sciences, University of Maribor, in nearby Hoče, Slovenia. Copper fungicides were applied to 8-year-old 'Golden Delicious' trees grafted on M9 rootstocks, planted at 0.7 m x 3.2 m tree-spacing. 'Golden Delicious' was chosen because it is a very russet-sensitive cultivar. TRV (tree row volume) was 12.000 m^3 and the maximum LAI (leaf area index) was 2.6. The trees were trained within a modified slender spindle-system. The experimental plots were arranged in a fully randomised block design with 4 replications. The plots were of approximately 300 m^2 (20m long and five rows wide). Only one row in the middle was sprayed with copper fungicides, other rows served as isolation against drift. The evaluations of copper phytotoxicity were also only performed in the middle row of trees. ANOVA analysis was performed for separation and comparison of the treatment means, followed by the use of multiple comparison tools based on Tukey's HSD test at ($P<0.05$).

Application of fungicides

The fungicides were applied using a Unigreen Turbo Teuton 5+5 standard orchard sprayer, which delivered 250 litres of spray per hectare. The droplet volume diameter (VMD) was 150-170 µm. The copper fungicides were applied 8 times per season. The rates of the fungicides per plot for all the tested formulations were adapted so that 250 grams of copper ions (Cu^{++}) per hectare were always applied. The cumulative amount of pure copper ions delivered per season per hectare amounted to 2000 g. The compositions of the tested copper fungicides are presented in Table 1. The copper fungicides were applied on May 23rd (fruit diameter 24-27 mm), June 2nd (FD 30-33 mm), June 10th (FD 35-40 mm), June 20th (FD 40-45 mm), June 27th (FD 50-55 mm), July 7th (FD 50-55 mm), July 29th (FD 55-65 mm), and August 5th (FD 65-70 mm). There were also two types of control plots. Control K1 – untreated by copper fungicides but treated 18 times with pesticides, and Control K2 – plots that were untreated with any of the preparations, neither with copper fungicides nor pesticides. At the times of applying the copper, the trees were always dry.

The K1 plots were sprayed 18 times a season with different fungicide and insecticide mixtures. They complied with the standard apple spray programme for the control of diseases and pests. We introduced these plots in order to compare the phytotoxicity levels of the copper fungicides with the level of phytotoxicity regarding the standard spraying programme. The following treatments were done at the K1 plots: 5th April (250 g thiram /ha; Tiram 80), 11th April (525 g dithianone / ha; Delan 700 WG), 17th April (200 g ciprodinyl /ha; Chorus 75 WG), 23rd April (75 g difenconazole / ha; Score 250 EC) + (400 g dodine /ha; Syllit 400), 30th April (75 g trifloxystrobin /ha; Zato 50 WG) + (1250 g captan /ha; Merpan) + (150 g tiacloprid /ha; Calypso), 10th May (same as 30th April), 18th May (same as 23rd April), 27th May (75 g flucvinconazol /ha; Clarinet) + (1250 g captan /ha; Merpan) + (90 g acetamprid / ha; Mospilan), 7th June (75 g krezoxim-methyl /ha; Stroby) + (2000 g sulphur /ha; Cosan) + (240 g tebufenozid /ha; Mimic), 20th June (525 g dithianone /ha; Delan 700 WG), 1st July (1250 g captan /ha; Merpan), 10th July (75 g trifloxystrobin ha; Zato), 20th July (1250 g captan /ha; Merpan) + (750 g chlorpyrifos /ha; Pyrinex), 28th July (2000 g sulphur /ha; Cosan), 8th August (200 g ciprodinyl /ha; Chorus 75 WG), 24th August (220 g boscalid /ha; Bellis), and 3rd September (200 g boscalid /ha; Bellis).

Table 1: Formulations of copper fungicides and additives tested during the trial

Formulation	Manufacturer	Chemical form of copper	g Cu^{++} / kg
Cuprablau Z ultra	Cinkarna d.o.o. SLO	Cu-Ca-oxychloride (nano particles)	350.0
Cinkarna EDTA	Cinkarna d.o.o. SLO	Cu-Ca- oxychloride-EDTA chelate (not fully chelated)	39.3
Coptrel 500	Yara Vita TM GB	Cu-oxide-urea complex	330.0
Copper Protein	Nova Prot GmbH DE	Cu-hydroxide-protein complex	18.0
Kupro 190 SC	Agroruše d.o.o SLO	Cu-oxo-sulphate	190.0
Peptiram 5	Sicit 2000 S.p.a. I	Cu-sulphate-peptide complex	50.0
Protex-CU	Ares Europe BV NL	water soluble Cu-sulphate	60.0
Nu-Film	Loffler AU	Conifer tree resin	
ProNet-alfa	Proagro GmbH DE	Protein based wetting agent	
Cutisan	Biofa DE	Kaolin clay	

Evaluation of phytotoxicity

For each experimental plot, samples of 150 fruits were randomly chosen from different parts of the tree-crown regions and the percentage area of fruit surfaces (%) with signs of phytotoxicity was visually determined (russet, deformation, changes of colour). The results are expressed as % of the russeted surface areas of the fruits. Each chosen fruit was observed very carefully from all sites in order to notice any change in structure of its peel. The percentage of fruit russeted surface area was calculated as an average mean value of 150 visually-estimated values for individual fruits. We used the same approach as is commonly used for estimating the percentage area of a diseased fruit's surface when studying fungicide efficacy (Laycock 2004). Russet estimation was carried out on the 29th August.

Analysis of copper content within the apples

In order to determine the copper content of the apples, the fruits were harvested at the end of September. Samples of 25 fruits were randomly chosen from different parts of trees from each experimental plot. Each fruit was manually rubbed using a plastic grater to obtain fruit pulp. The fruits were unwashed prior to rubbing. The preparation of the samples for the analysis of copper content was performed according to the procedure established by the Cinkarna Metallurgical and Chemical Industry Celje, Inc.. The pulp samples were mineralised in microwave (nitric acid + hydrogen peroxide decomposition procedure), filtrated, and the solutions prepared for the analysis carried out by an Atomic Absorption Spectrometer (Varian AA 240FS), and by reading of their absorbance at 324.8 nm.

RESULTS AND DISCUSSION

Phytotoxicity analysis

The results presented in Table 2 demonstrate that different copper fungicides have a significantly different level of phytotoxicity to apple fruits, and that addition of additives results in different levels increasing or decreasing of phytotoxicity. Systemic acting fungicides (Peptiram, Protex and Copper Protein) caused a significantly higher level of russet than contact acting ones (Cuprablau and Cupro) (see last column of Table 2). The addition of Nu-Film, on average, reduced the level of russet from 5.40% to 4.11%, significantly less than in the case of adding Cutisan (from 5.40% to 3.29%). The addition of ProNet-alfa, on average, increased the russet area of fruits (from 5.40% to 5.73%). These results demonstrate how interactions amongst copper fungicides and additives can differ and that certain additives are unsuitable for mixing with certain copper fungicides. For example, it was obvious that ProNet-alfa is unsuitable for mixing with Cuprablau (russet increased from 1.99% to 6.29%). The highest reduction of russet was achieved by adding kaolin clay. The results show that kaolin clay is the most suitable additive for reducing copper fungicide phytotoxicity from amongst the three tested.

In the literature we were unable to find extensive data about those additives that can be used for the reduction of copper fungicide phytotoxicity. During our previous research we established that Cutisan (kaolin clay) provides a considerable level of reduction in phytotoxicity (Lešnik et al. 2011). We found the reports of Privé and co-workers (2007) who established that adding of kaolin clay can reduce copper phytotoxicity to apples significantly, but not during all seasons. According to their opinion, the russet phenomenon is very much weather-related and that during certain weather conditions the addition of additives cannot prevent russet. The same was proven by Noè and Eccher (1996). They didn't, however, test additives similar to ones we tested. Researchers De Jong and Der Maas (2008) studied the russet of Elstar apples and discovered that copper treatments were less problematic in terms of phytotoxicity than treatments with Armicarb (potassium bicarbonate). Potassium bicarbonate fungicides are one of the important alternative products by which the replacement of using copper fungicides during organic production is usually carried out. They found out that copper phytotoxicity can be reduced by mixing copper fungicides with sulphur fungicides. They mentioned the possibility of adding kaolin clay but didn't present any results. Similar results on the effects of adding sulphur were determined by Mitre and Mitre (2009), but they obtained opposite results about the phytotoxicity effects of Armicarb. According to their opinion, the mixing of copper products with potassium bicarbonate can slightly reduce copper fungicide phytotoxicity. In the literature there are many manuscripts available on methods for the reduction of russet by the application of hormones (especially GA4 and GA7 gibberellins) (Taylor and Knight 1986, McArtney and Obermiller 2007). These kinds of solutions are incompatible with Slovenian-integrated and organic fruit production regulations.

In regard to Golden delicious apples, a certain level of russet is inevitable and it also appears on fruits that have been untreated with any agrochemical. During our trial we had two types of control plots. Fruits from the K1 control plots that were sprayed 18 times a season with organic fungicides and insecticides but were not sprayed with any of the copper products, also had a certain level of russet (0.3 – 2.9%). This means that organic pesticides cause a certain level of phytotoxicity. There were also control plots (K2) that were not sprayed with any of the copper fungicides, additives or standard pesticides. Russet at those plots, on average, amounted to 1.25% (from 0.2 to 2.1%), and was the result of unknown environmental and physiological factors. We cannot define which of the factors having effects on the level of russet, and known from the literature (Knoche at al. 2011; e.g. daily temperature and air humidity regime, fertilisation rate nutrients, clone-type and age of trees), played an important role during the period of our trial. According to our measurements of weather parameters, as the meteorological conditions during the summer period of 2011 didn't fluctuate much from the data from 30-year local meteorological average values.

Table 2: The rate of the fruit damage (% of the russeted and malformed surface areas of fruits) in relation to the copper fungicides and additives applied. Assessment on August 29th

Fungicide	No additives	Nu-Film	Cutisan	ProNet-alfa	Average
Cuprablau Z ultra	1.99 a A	1.41 ab A	1.17 a A	6.29 c B	2.85 a
Cinkarna EDTA	2.23 a A	1.77 b A	4.09 c A	2.00 ab A	2.52 a
Peptiram 5	9.22 c C	5.83 cd B	1.78 ab A	9.41 c C	6.72 c
Coptrel 500	5.54 b B	4.97 c AB	2.42 b A	5.35 bc B	4.57 b
Protex-CU	4.03 b AB	3.59 c A	2.84 b A	5.05 bc B	4.01 b
Kupro 190 SC	3.26 ab B	3.45 c B	1.88 ab A	3.24 b B	2.96 a
Copper Protein	9.02 c B	7.75 d A	6.88 d A	9.25 c B	8.17 d
Control K1	2.61 a B	0.80 a A	0.60 a A	1.12 a A	
Average*:	5.04 C	4.11 B	3.29 A	5.73 D	

* Control K1 excluded from calculation of average mean. Control K2 -1.25%.
** Means marked with the same letters do not differ significantly according to the Tukey HSD test (P<0.05). The capital letters serve for comparisons amongst additives and the small letters for comparisons amongst fungicides.

Table 3: Copper content of apple fruits (mg/kg fresh weight) in relation to the copper fungicides and additives applied. Assessment on September 28th

Fungicide:	No additives	Nu-Film	Cutisan	ProNet-alfa	Average
Cuprablau Z ultra	2.31 ab A	4.87 c B	3.39 b A	3.28 b A	3.46 b
Cinkarna EDTA	1.55 a A	1.90 ab A	2.00 abA	2.71 b A	2.04 a
Peptiram 5	2.52 b A	2.50 b A	2.06 ab A	2.63 ab A	2.43 a
Coptrel 500	1.93 a A	2.82 b A	2.20 ab A	2.31 ab A	2.31 a
Protex-Cu	3.03 b A	3.80 bc B	2.15 ab A	3.25 b A	3.06 b
Kupro 190 SC	1.46 a A	2.18 b AB	2.55 b B	1.70 a A	1.97 a
Copper Protein	1.91 a A	2.02 ab A	2.63 b B	2.51 ab AB	2.27 a
Control K1	1.79 a A	1.35 a A	1.25 a A	1.48 a A	
Average*:	2.21 A	2.76 B	2.42 AB	2.63 AB	

* Control K1 excluded from the calculation of average mean. Control K2-1.74 mg/kg.
** Means marked with the same letters do not differ significantly according to the Tukey HSD test (P<0.05). The capital letters serve for comparisons amongst additives and the small letters for comparisons amongst fungicides.

Copper content analysis

Additives influence copper ion adhesion to fruit surfaces and penetration into fruit tissues (Teviotdale and Viveros 1999, Orbovič et al. 2007). The aim of our study was to determine the relationship between the metallic copper concentrations of fruits and the level of phytotoxicity regarding copper fungicides. Data on the copper contents of fruits is presented in Table 3. We can see that there were some differences in the contents of copper in those fruits treated with different fungicides. When additives were omitted, statistically the differences amongst the tested fungicides were insignificant. Those fruits treated with systemic-acting fungicides (Protex and Peptiram) contained more copper than those fruits treated with contact-acting fungicides (Cuprablau, Kupro, and Coptrel). The addition of Nu-Film increased the copper content of the fruits for all copper fungicides from 2.21 mg/kg to 2.76 mg/kg. A 24.8% increase was determined, on average. This result can be explained by the Nu-Film features. Nu-Film is a resin-based additive aimed at stabilising spray deposit and extending its lifespan. Copper deposits on the fruits' surfaces remain for longer periods and the penetration of copper ions is not enhanced. The addition of Cutisan also slightly increased the copper content of the fruits (from 2.21

mg/kg to 2.42 mg/kg; 9.5% increase), but insignificantly. The increase caused by adding ProNet-alfa amounted to 19.0% (from 2.21 mg/kg to 2.63 mg/kg).

The results show that the important side-effect of adding additives is the increase of copper content in the fruits. The results do not allow us to declare any conclusive relationship between the increased copper content and the increased level of phytotoxicity, as the increased content of copper does not directly correlate with the increased level of phytotoxicity. For example, this can be seen in the results for Copper Protein when comparing data from Table 2 and 3 – no additives). The phytotoxicity of Copper Protein decreased (from 9.02% to 7.75%) by the addition of Nu-Film, despite the increase in copper content (from 1.91 to 2.02 mg/kg). Similar results were noticed in the case of Cuprablau. The phytotoxicity of Cuprablau expressed as russet was decreased from 1.99% to 1.42% by adding Nu-Film despite the increase of copper content (from 2.31 to 4.87 mg/kg). The rate of proportionality between the level of reduced russet and the level of copper content was the highest in the case of Cutisan. The addition of Cutisan decreased the level of phytotoxicity the most and increased the copper content the least of all three of the tested additives. In the literature there is very limited information available on the effects of additives on the penetration of copper ions into apple fruit tissues, therefore we cannot directly compare our results with the results of other researchers.

CONCLUSIONS

The effects of adding additives to copper fungicide sprays in order to achieve a reduction in phytotoxicity for apple fruits are quite unpredictable. Some copper fungicides have shown that they are less likely to cause phytotoxicity than others. With certain combinations of fungicides and additives, significant reduction of phytotoxicity can be achieved, but not with all, because in some cases the addition of additives can result in increased phytotoxicity.

Every combination of copper fungicide and additive needs to be tested during a small-scale trial before advised for broader use. According to our results, kaolin clay is the most suitable additive for the reduction of phytotoxicity regarding the copper fungicides tested. The determined reduction level (from 19 to 35%) was not sufficiently high enough for us to conclude that any addition of the tested additives can solve the phytotoxicity problem completely.

It is also important to take into account that the usages of certain additives can increase the intake of copper ions into apple fruit, which can result in an increased, or even too high copper content of the fruits.

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