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Seasonal variation in extracted proteases and relationship to overall soil protease and exchangeable ammonia in paddy soils

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Abstract In paddy fields treated with organic manure or chemical fertilizer or not treated, seasonal changes in soil protease [hydrolysis to benzyloxycarbonyl-L-phenylalanyl-L-leucine (z-FLase) or casein (caseinase)] extracted with phosphate buffer were investigated during the cultivation of rice. Both activities reached a maximum on July 21 (after irrigation), and gradually decreased, which was correlated with the soil water content. Increases in z-FLase and caseinase activity extracted coincided with increases in exchangeable NH_4^+ and the ninhydrin-reactive N content. The overall soil caseinase activity on August 16 (after midsummer drainage) was inhibited by metal chelators and *p*-chloromercuribenzoic acid and was similar to that extracted with 1.0 mol l^{-1} phosphate buffer on June 9 (before irrigation), but different from that extracted by 0.1 mol l^{-1} phosphate buffer on July 22. The overall soil z-FLase activity on August 16 was inhibited by *p*-chloromercuribenzoic acid and 1,10-phenanthroline, and was similar to that extracted with 0.1 mol l^{-1} phosphate buffer on June 9, but differed from that extracted on July 22. The results indicate that the soil proteases extracted on July 22 had a high potential for ammonification and were short lived.

Key words Extracted soil z-FLase · Extracted soil caseinase · Exchangeable NH_4^+ · Ninhydrin-reactive nitrogen · Soil protease · Wetland rice · Crop rotation

Introduction

Proteases have been extracted from soil in order to investigate the relative extraction efficiency (Ladd 1972; Nannipieri et al. 1980), and the properties (Ceccanti et al. 1989), types (Ladd 1972; Mayaudon et al. 1975; Hayano et al. 1987; Hayano and Watanabe 1990; Hayano 1993; Watanabe and Hayano 1993 b), and origins of the immobilized enzymes (Ladd 1972; Watanabe and Hayano 1993 b).

In soil, free enzymes released by soil microorganisms can be easily extracted but they are easily decomposed by autolysis or microbial degradation (Burns 1982) unless they are bound to soil colloids, clay, and humus. It is thought that after binding they can persist for a long time in soil (Paulson and Kurtz 1969; Sarkar et al. 1989), although their activity decreases (Zvyagintsev et al. 1987; Lähdesmaki and Piispanen 1992). It has been proposed that soluble soil protease is newly synthesized by microbial cells (Asmar et al. 1992). In paddy fields, where the soil has a high water content throughout the rice cultivation period, soluble soil proteases might be effectively hydrolyzed and make an important contribution to the ammonification process. There have been no reports on the relationship between extracted soil protease, total soil protease, and the ammonification process, in paddy soil. In the present study seasonal changes in extracted soil proteases in paddy field and their relationship to total soil proteases were assessed. In addition, their properties, relationship to the ammonification process, and source were examined. A pyrophosphate buffer is more efficient than a phosphate buffer in extracting enzymes intimately bound or immobilized by soil colloids (Nannipieri et al. 1980). We used a phosphate buffer to extract easily solubilized proteases (Hayano et al. 1987).

Materials and methods

Chemicals

Benzyloxycarbonyl-L-phenylalanyl-L-leucine (z-FL) and casein were used as substrates for protease. The hydrolytic activity on each substrate was defined z-FLase and caseinase, respectively. z-FL was purchased from Sigma Chemical Co. Hammarsten casein and the

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ninhydrin reagent L-8500 set were purchased from Wako Chemical Co.

Soils

Non-rhizosphere soil samples (depth 0–15 cm) from three paddy fields in Chikugo, Japan (Gray Lowland soil), under a rice and wheat rotation were collected over 2 years, on July 22 (after irrigation), August 12 (after midsummer drainage), and October 21 (after the final drainage) in 1991, and on June 9 (before tillage), July 21 (after irrigation), August 16 (after midsummer drainage), September 16 (before drainage), and October 13 (after the final drainage) in 1992. After harvesting wheat (June 1), the fields were tilled (June 9), fertilized and irrigated (June 17), and then rice was planted and cultivated; for this season the fields were flooded from June 10 to July 27, and then drained until August 4 (midsummer drainage); after drainage the fields were irrigated intermittently until 2 weeks before harvest. There were three experimental fields, each with a different fertilizer treatment. The fertilizer treatments were (1) an annual application for 15 years of rice-straw compost (20 t ha^{-1}; before irrigation) and chemical fertilizer (90 kg NH_3 ha^{-1}) on June 17 (50 kg), August 12 (20 kg), and August 20 (20 kg); (2) annual treatment with chemical fertilizer (130 kg NH_3 ha^{-1}) for 4 years, on June 17 (60 kg), July 17 (30 kg), August 17 (20 kg), and August 31 (20 kg); and (3) no treatment for 4 years. After sampling, the soil was sieved (<2 mm) and used immediately for the experiments.

Total soil protease activity

Total soil z-FLase and caseinase activities were measured using the method described by Watanabe and Hayano (1993b). z-FLase was measured after 5 h of incubation of a soil suspension of z-FL (1 mmol l^{-1}) in Atkins-Pantin buffer (pH 8.0, 0.1 mol l^{-1}). The amount of leucine liberated was determined by the absorbance at 570 nm following reaction with ninhydrin reagent. Caseinase activity was measured by changing the Atkins buffer to a phosphate buffer (pH 7.0, 50 mmol l^{-1}) and z-FL to casein (0.6% in phosphate buffer). Caseinase activity was expressed as the leucine equivalent. Enzyme activity was defined as the amount (1 pKat) of enzyme liberating 1 pmol leucine (for z-FLase) or leucine equivalent (for caseinase) s^{-1} in our experimental conditions.

The effect of inhibitors on the soil suspension was examined by adding 0.6 ml of the inhibitor solution (50 mmol l^{-1} in dimenthylsulfoxide) to the soil suspension. The inhibitory effect was expressed as a percentage of the rate observed in the presence of the inhibitor compared with the activity without the inhibitor.

Extraction of protease

Soil proteases were extracted by the method described by Hayano et al. (1987). Freshly collected moist soil (10 g) was extracted with 20 ml phosphate buffer (0.1 and 1.0 mol l^{-1}), pH 7, by shaking (260 oscillations min^{-1}, 5.0 cm stroke length) for 30 min at 30 °C. The supernatant was separated by centrifugation (20 min at 9000 r min^{-1}, rotor size $\varnothing$ = 188 mm). The extraction was repeated once. The combined supernatant was treated with solid ammonium sulfate (20 g) and incubated at 4 °C. After 3 h, the precipitate was collected by centrifugation (10 min at 9000 r min^{-1}) and dissolved in 3 ml distilled water. The solution was dialyzed overnight against cold distilled water at 4 °C, and used as an enzyme solution. Extractable soil z-FLase and caseinase activity and the effects of the inhibitors were measured using the method described previously (Watanabe and Hayano 1993b).

Ninhydrin-reactive N

The ninhydrin-reactive N content was determined by the method described by Amato and Ladd (1988). Freshly collected moist soil (20 g) was extracted with 100 ml KCl solution (2 mol l^{-1}) by shaking for 30 min at 30 °C. Supernatant was decanted and 2 ml supernatant was treated with 1 ml buffer solution and ninhydrin reagent L-8500 and boiled for 20 min. The mixture was rapidly cooled in an ice-water bath, and 3 ml 50% ethanol was added. The absorbance at 570 nm was measured in a spectrophotometer and ninhydrin-N was expressed as the leucine equivalent. Under the conditions described, color responses for leucine and NH_4^+ are similar and 93 and 87% of the maximum color yields, respectively, were obtained (unpublished results). Ninhydrin-N represents the sum of α-amino N, primary mines, and NH_4^+, as suggested by Joergensen and Brookes (1990).

Exchangeable NH_4^+

Exchangeable NH_4^+ in the extracted KCl solution (50 ml) was measured by steam distillation with MgO as described by Keeney and Nelson (1982).

Correlation and clustering procedure

The relationship among the factors was evaluated by correlation analysis. For this purpose we used the seasonal data presented in this paper (soil z-FLase and caseinase activity extracted with 0.1 mol l^{-1} phosphate buffer measured in 1991 and 1992; contents of ninhydrin-N and exchangeable NH_4^+ in 1992) and data from Watanabe and Hayano (1995) on overall soil z-FLase and caseinase activity, total numbers of *Bacillus* spp., their vegetative cells and spores measured in 1992; soil water contents in 1991 and 1992; and numbers of total bacteria, and proteolytic bacteria counted on azocoll albumin agar plates and azocoll nutrient agar plates, estimated by a most probable number method using 12 and 15% gelatin media in 1991.

The similarity between the extracted and the overall soil protease activity was evaluated using cluster analyses on the basis of the effects of inhibitors. For this purpose we used the data presented in this paper (overall soil z-FLase and caseinase activity determined on August 16; z-FLase extracted by 0.1 mol l^{-1} phosphate buffer on August 16; soil caseinase extracted by 1.0 mol l^{-1} phosphate buffer on June 9), and data on extracted protease activity from Watanabe and Hayano (1993b), comprising soil z-FLase and caseinase extracted by 0.1 mol l^{-1} phosphate buffer on July 22; soil z-FLase extracted by 0.1 mol l^{-1} phosphate buffer on June 9). Clustering was achieved using the group average method with the standardized squared Euclidean distance.

Results

Seasonal variation in extracted soil protease activity

In 1992, ratios between extracted and overall total soil z-FLase activity (Watanabe and Hayano 1995) were invariably higher with 0.1 than 1.0 mol l^{-1} phosphate buffer in the three paddy fields (Table 1). These ratios reached a maximum on July 21, and then gradually decreased to an almost undetectable level on October 13 (Table 1). In the three fields, soil z-FLase activity extracted with 0.1 mol l^{-1} phosphate buffer showed similar variations in 1991 and 1992 (Fig. 1).

Table 1 Seasonal variation in ratios between extracted and overall benzyloxycarbonyl-L-phenylalanyl-L-leucinase (z-FLase) activity soil (Watanabe and Hayano 1995). Extraction was by 0.1 or 1.0 mol l^{-1} phosphate buffer (*PB*) at pH 7.0. Soils: *OM* annual application of rice-straw compost plus split application of N fertilizer, *CF* split application of N fertilizer, *NF* no fertilization (– not detectable, *ND* not determined)

Date	Paddy soils (PB)					
	OM		CF		NF	
	0.1 *M*	1.0 *M*	0.1 *M*	1.0 *M*	0.1 *M*	1.0 *M*
June 9	10.4%	6.8%	12.8%	6.7%	7.0%	1.3%
July 21	90.6%	15.7%	57.9%	1.3%	40.0%	1.2%
August 16	27.6%	18.4%	29.5%	18.0%	23.6%	24.6%
September 16	11.1%	–	2.7%	–	24.6%	–
October 13	ND	–	0.8%	–	0.3%	–

Ratios between the extracted and the overall soil caseinase activity (Watanabe and Hayano 1995) in 1992 (Table 2) were smaller than those for z-FLase (Table 1). In the three fields, soil caseinases were extracted on June 9 only by 1.0 mol l^{-1} phosphate buffer and on July 21 only by 0.1 mol l^{-1} phosphate buffer (Table 2). Less soil caseinase activity was extracted with 0.1 mol l^{-1} phosphate buffer than z-FLase activity but the patterns of fluctuation were similar for each enzyme (Figs. 1, 2).

Seasonal variation in ninhydrin-reactive N and exchangeable NH_4^+

In the two fertilized fields, the ninhydrin-N-content increased after irrigation (July 21) and remained at a low level until October 13 (Fig. 3). In the unfertilized field, it increased further on September 16 (Fig. 3).

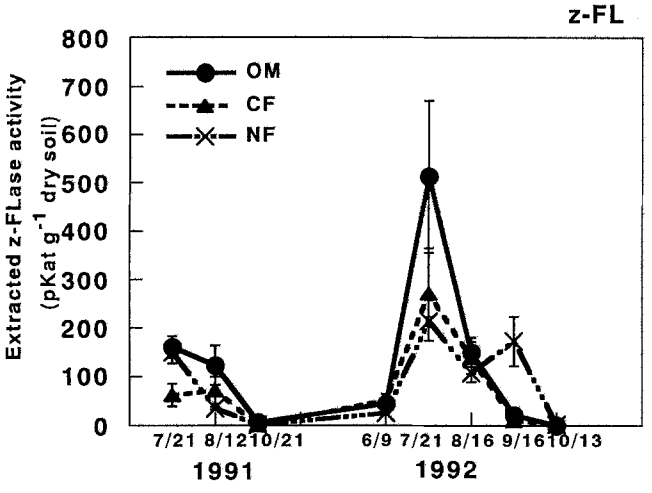

Fig. 1 Seasonal variation in benzyloxycarbonyl-L-phenylalanyl-L-leucinease or hydrolysing (z-FL) activity extracted from soil with 0.1 mol l^{-1} phosphate buffer, pH 7.0. *Bar* indicates SD; *OM* soil treated annually with organic matter plus split application of fertilizer N, *CF* N split application of fertilizer, *NF* no fertilization

Table 2 Seasonal variation in ratios between extracted and overall caseinase activity (see Table 1 for further explanations)

Date	Paddy soils (PB)					
	OM		CF		NF	
	0.1 *M*	1.0 *M*	0.1 *M*	1.0 *M*	0.1 *M*	1.0 *M*
June 9	ND	9.8%	ND	7.6%	ND	2.1%
July 21	18.0%	ND	2.6%	ND	1.3%	ND
August 16	ND	ND	ND	ND	ND	ND
September 16	ND	–	ND	–	ND	–
October 13	0.6%	–	0.8%	–	ND	–

Exchangeable NH_4^+ values showed similar variations to those of ninhydrin-N with about twofold higher values. The values increased after irrigation (July 21) and remained at a high level until October 13 in the two fertilized fields (Fig. 3). In the unfertilized field, it increased further on September 16 (Fig. 3).

Characterization of extracted and overall soil protease

The overall z-FLase activity in three paddy soils on August 16 was moderately inhibited by 1,10-phenanthroline and *p*-chloromercuribenzoic acid (Table 3). The z-FLase extracted with 0.1 mol l^{-1} phosphate buffer on August 16 was highly inhibited by 1,10-phenanthroline and iodoacetic acid, and moderately by 3-phenylpropionic acid, ethylenediaminetetraacetic acid (EDTA) and *p*-chloromercuribenzoic acid (Table 3).

The overall soil caseinase activity in three paddy fields on August 16 was moderately inhibited by EDTA, 1,10-phenanthroline and *p*-chloromercuribenzoic acid (Table 4). Caseinase activity extracted with 1.0 mol l^{-1} phosphate buffer on June 9 was highly inhibited by

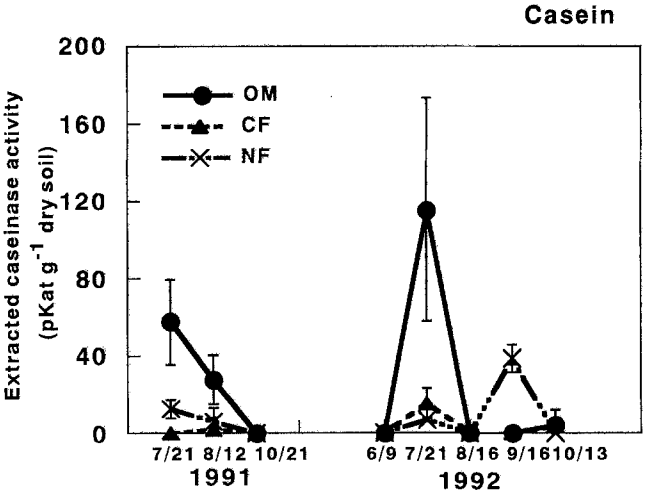

Fig. 2 Seasonal variation in soil caseinase activity extracted from soil with 0.1 mol l^{-1} phosphate buffer, pH 7.0 (see Fig. 1 for further explanations)

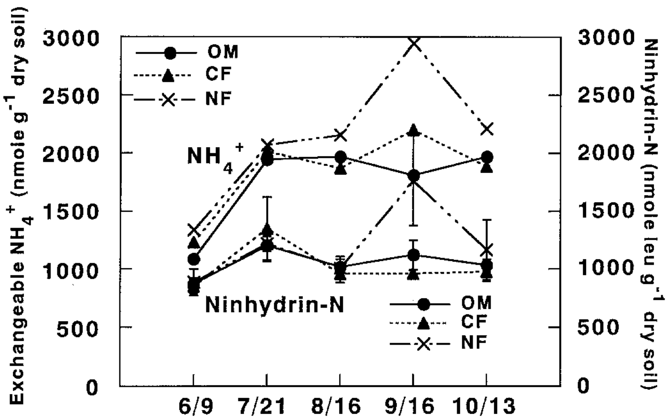

Fig. 3 Seasonal variation in ninhydrin-N and exchangeable NH_4^+ (see Fig. 1 for further explanations)

Table 3 Effect of inhibitors (2.5 mmol l^{-1} for the soil suspension and 0.5 mmol l^{-1} for extracted soil protease) on overall and extracted soil z-FLase activity on August 16. Values are expressed as the percentage of control without inhibitor (*EDTA* ethylenediaminetetraacetic acid, *1,10P* 1,10-phenanthroline, *pCMB* p-chloromercuribenzoic acid, *IAA* iodoacetic acid, *PMSF* phenylmethylsulfonyl fluoride, *DFP* diisopropylfluorophosphate, *PPA* 3-phenylpropionic acid, *Peps* pepstatin; see Table 1 for further explanations)

	EDTA	1,10P	pCMB	IAA	PMSF	DFP	PPA	Peps
The overall soil protease								
OM	102	63	58	80	95	87	76	88
CF	107	54	47	68	90	77	72	97
NF	121	41	55	78	86	85	76	104
Protease extracted by 0.1 mol l^{-1} phosphate buffer								
OM	55	9	50	33	85	94	51	85

Table 4 Effect of inhibitors on overall (August 16) and extracted (June 6) soil caseinase activity (see Tables 1 and 3 for further explanations)

	EDTA	1,10P	pCMB	IAA	PMSF	DFP	PPA	Peps
The overall soil protease								
OM	63	66	59	102	86	90	105	104
CF	64	71	58	95	81	76	95	84
NF	47	74	63	83	80	77	91	88
Extracted soil protease by 1.0 mol l^{-1} phosphate buffer								
OM	67	28	75	79	70	110	103	83
CF	64	7	67	74	83	87	99	74

1,10-phenanthroline and moderately by EDTA and p-chloromercuribenzoic acid.

Discussion

We have suggested that proteolytic *Bacillus* spp. might be the major source of soil protease in paddy fields under a rotation of rice and wheat because these bacteria are widely proliferated in these paddy fields (Watanabe and Hayano 1993a) and they produce large amounts of extracellular protease (Watanabe et al. 1994) with similar properties to the extracted soil proteases (Watanabe and Hayano 1993b). Correlation analyses of seasonal data for overall soil protease, numbers of proteolytic bacteria, and *Bacillus* spp. cells suggested that the overall soil z-FLase might be concerned with the number of proteolytic bacteria and the overall soil caseinase might be concerned with *Bacillus* spp. spores (Watanabe and Hayano 1995). However, the relationship between the extracted and the overall soil proteases and their contributions to the ammonification process have remained unclarified.

In 1992, z-FLase extracted with 0.1 mol l^{-1} phosphate buffer was significantly correlated with soil caseinase extracted similarly ($r = 0.85$; $P<0.01$, $n = 15$) and weakly with soil moisture values reported previously (Watanabe and Hayano 1995) ($r = 0.46$, $P<0.1$; Table 5). In 1991, the extracted z-FLase activity was also positively correlated with the extracted caseinase activity ($r = 0.721$, $P<0.05$, $n = 9$; Table 6) and both extracted protease activities were positively correlated with soil moisture (z-FL, $r = 0.78$, $P<0.05$; casein $r = 0.72$, $P<0.05$; Table 6). The extracted protease activity was not significantly and positively correlated with numbers of vegetative cells and spores of *Bacillus* spp. nor the overall soil protease activity (Table 5) nor numbers of total and proteolytic bacteria reported previously (Watanabe and Hayano 1995). The results differed from those of an incubation test (Asmar et al. 1992).

The ninhydrin-N content was correlated (Table 5) with the exchangeable NH_4^+ content ($r = 0.77$, $P<0.01$) and weakly with the extracted protease activity (z-FLase, $r = 0.48$, $P<0.1$; caseinase, $r = 0.45$, $P<0.1$), soil moisture ($r = 0.48$, $P<0.1$), and overall soil z-FLase activity ($r = 0.48$, $P<0.1$) reported by Watanabe and Hayano (1995). The exchangeable NH_4^+ content was correlated

Table 5 Correlation coefficients between numbers of *Bacillus* spp. soil moisture, exchangeable NH_4^+, ninhydrin-N (*Nin-N*), and extracted and overall protease activity ($n = 15$). *z-FL* total soil z-FLase activity, *Casein* total soil caseinase activity, *PP* total numbers of *Bacillus* spp. counted on peptone polymyxin medium, *BTV* numbers of vegetative cells of *Bacillus* spp. counted on *Bacillus thuringiensis* medium, *PP80* numbers of spores of *Bacillus* spp. counted on peptone polymyxin medium after 30 min 80 °C incubation, *Moist* soil moisture in 1992 as described by Watanabe and Hayano (1995); positive correlations significant at 10%*, 5%**, and 1%***

	Casein	NH_4^+	Nin-N	z-FL	Casein	PP	BTV	PP80	Moist
z-FL	0.85***	0.23	0.48*	0.32	0.29	−0.13	0.04	0.08	0.46*
Casein		0.24	0.45*	0.35	0.08	−0.15	0.01	−0.10	0.26
NH_4^+			0.77***	0.54**	−0.31	−0.68	−0.44	−0.38	0.65***
Nin-N				0.48*	−0.28	−0.33	0.01	−0.50	0.48*

Table 6 Correlation coefficients between numbers of proteolytic bacteria, soil moisture, and extracted protease activity ($n = 9$); *z-FL* z-FLase (Table 1) activity; *AA* total number of bacteria counted on albumin agar plates, *AAA* proteolytic bacteria counted on azocoll albumin agar plates, *ANA* proteolytic bacteria counted on azocoll nutrient agar plates; *MPN12* proteolytic bacteria estimated from most probable number method using 12% gelatin medium, *MPN15* proteolytic bacteria estimated from most probable number method using 15% gelatin medium, *Moist* soil moisture in 1991 described by Watanabe and Hayano (1995); positive correlations significant at 10%*, 5%**, and 1%***

	Casein	AA	AAA	ANA	MPN12	MPN15	Moist
z-FL	0.72**	−0.67	−0.12	−0.40	−0.67	−0.31	0.78**
Casein		−0.64	−0.18	−0.57	−0.36	−0.33	0.72**

(Table 5) with soil moisture ($r = 0.65$, $P < 0.01$), and the overall soil z-FLase activity ($r = 0.54$, $P < 0.05$).

The data suggested that the proteases extracted on July 21 had a higher potential for ammonification than total soil protease in the paddy fields because (1) the increase in extracted soil protease activity (July 21 in all three fields and September 16 in the unfertilized field; Figs. 1 and 2) coincided with the increase in ninhydrin-N content and exchangeable NH_4^+ (Fig. 3), although we cannot give a reliable explanation for the higher exchangeable NH_4^+ than ninhydrin-N content, and (2) extracted protease is probably loosely bound and comes into contact with the substrate more effectively than the tightly bound enzymes (Zvyagintsev et al. 1987; Lahdesmaki and Piispanen 1992); for example, 200 pKat of extracted soil protease activity (Fig. 1) was sufficient to produce about 2000 nmol g^{-1} of NH_4^+ in 3 h, which corresponded to the increase in NH_4^+ on July 21 in all three fields, and on September 16 in the unfertilized soils (Fig. 3). This observation agrees with incubation results (Asmar et al. 1994).

The relationships between properties of the extracted and the overall soil z-FLase are represented on a dendrogram (Fig. 4). Dissimilarities are clustered in two defined groups, with all of the overall soil z-FLase on August 16 belonging to the same cluster as the extracted soil z-FLase on June 9 reported by Watanabe and Hayano (1993b). In the dendrogram for the extracted and overall soil caseinases, all of the overall soil caseinase on August 16 also belong to the same cluster as the extracted caseinase on June 9 (Fig. 5). The overall soil z-FLase and caseinase on August 16 were similar to the extracted protease on June 9, but not to the proteases on July 22 reported by Watanabe and Hayano (1995). From these results, we concluded that the soil protease extracted on July 22 comprised a major part of the total soil protease but might be short-lived due to rapid decomposition. This hypothesis is in agreement with results obtained by Asmar et al. (1994).

The higher extracellular z-FLase and caseinase activities observed on July 21 compared with the other sampling days might have been caused by the release of protease from protease-soil colloid complexes. This hypothesis is based on the observations that (1) both extracted z-FLase and extracted caseinase activities were correlated with soil moisture, except for caseinase in 1992, and (2) the extracted proteases were not correlated with overall soil protease activities, nor with numbers of proteolytic

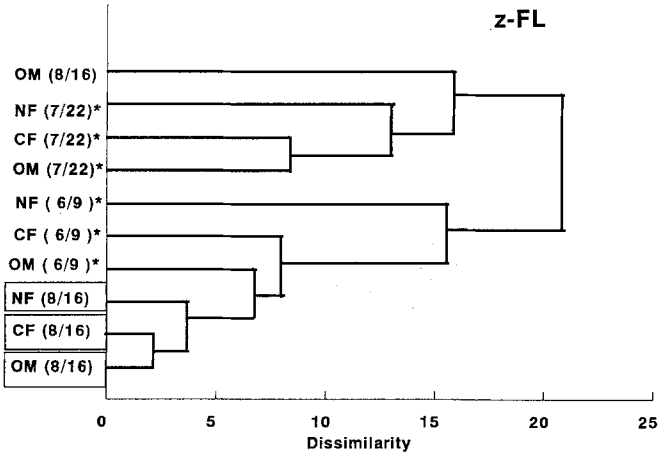

Fig. 4 Dendrogram from group average method of extracted and the overall soil z-FLase (in *boxes* shown with month and day). Dissimilarity was standardized squared Euclidean distance. *Data presented in Watanabe and Hayano (1993b) were used. See Fig. 1 for further explanations

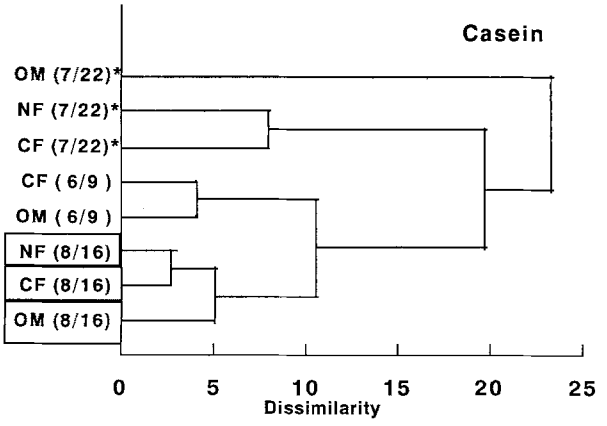

Fig. 5 Dendrogram from group average method of extracted and overall soil caseinase (in *boxes* shown with month and day). Dissimilarity was standardized squared Euclidean distance. *Data presented in Watanabe and Hayano (1993b) were used. See Fig. 1 for further explanations

bacteria or *Bacillus* spp. However, we could not exclude the possibility of an increase in de novo synthesis in response to the change in environmental conditions because the protease extracted on July 22 had different enzymatic characteristics from those of the June 9 protease and the overall proteases (Tables 4, 5). We propose that specific proteolytic *Bacillus* spp. were involved in the increase in extracted protease because numbers of total bacteria and *Bacillus* spp. decreased after irrigation (Watanabe and Hayano 1994, 1995), whereas the proteolytic *Bacillus* spp. was largely isolated after irrigation, which liberated larger amounts of extracellular proteases with similar properties to those of the extracted soil z-FLase (Watanabe and Hayano 1993 b) than the other bacteria and *Bacillus* spp. isolated in the other seasons (Watanabe and Hayano 1993 a; Watanabe et al. 1994). These proteolytic *Bacillus* spp. might proliferate or produce extracellular protease after irrigation when more substrate is available from killed cells of aerobic microorganisms. However, they form only a small proportion of the total soil bacteria and cannot be counted by known detection methods.

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