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FIRST REPORT OF B.N. *RHIZOCTONIA* FROM TOBACCO (*NICOTIANA TABACUM* L.) IN SAMSUN, TURKEY

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Abstract

In this study, 14 fungal samples were isolated from tobacco (*Nicotiana tabacum* L.) cultivated in Samsun-Turkey, corresponded with binucleate *Rhizoctonia* (BNR) anamorphic criteria. Of these, 10 isolates were identified as BNR AG-A, one as BNR AG-Fa and 3 as BNR AG-Fb at the results of anastomosis tests and rDNA-ITS phylogenies. Five of 10 AG-A isolates were found as inducers for root rot of tobacco, whereas 5 AG-A isolates and AG-Fa, Fb isolates were found as slightly virulent or non-pathogenic. This study presents the first report for B.N. *Rhizoctonia* AG-A as a pathogen for tobacco.

Introduction

Binucleate *Rhizoctonia* spp., (Teleomorph: *Ceratobasidium* spp., and *Tulasnella* spp.) have been reported as inducers of diseases like damping-off, root rot, stem rot, sheath blight, fruit decay and foliar blight on a wide variety of important agricultural plants and some of them also live as saprophytic or as mycorhiza (Hyakumachi *et al.*, 2005). They (except *R. repens*) are divided into anastomosis groups (AGs) according to the capability of their hyphae to fuse with members of designated anastomosis groups (Sneh *et al.*, 1991). Currently 16 BN *Rhizoctonia* AGs (A to S) are known because, some AGs were excluded from BN *Rhizoctonia* (J and N), some were lost and also some does not exist in any culture collection (M) or transferred to other AGs (T to A and U to P) of BN *Rhizoctonia* (Sharon *et al.*, 2008).

Tobacco is one of the most important agricultural plant for both Turkey and province of Samsun. The cultivation areas of tobacco in Turkey and Samsun were 194.683 ha and 18.234 ha, respectively in 2002; indicating that Samsun is one of most important districts of Turkey for tobacco cultivation (Anon., 2004). Tobacco has been reported to be infected by *R. solani* AGs 1 through to 5 which causes diseases like damping-off, sore shin and leaf target spot of tobacco (Csinos & Stephenson, 1999; Nicoletti *et al.*, 1999; Tarantino *et al.*, 2007) and recently BN *Rhizoctonia* AG-G and AG-R were also reported as damping-off inducers for Tobacco (Garcia *et al.*, 2009). At current study, after our last research where we reported *R. solani* AGs 1-IB, 2-1, 4 HG-I and 4 HG-II as pathogens of tobacco in Samsun/Turkey (Gurkanli *et al.*, 2009), we aimed to characterize the BN *Rhizoctonia* isolates infecting tobacco in the same locality.

Materials and Methods

Sampling, isolation and identification of BN *Rhizoctonia* isolates: The samplings for tobacco plants were made in 9 districts of Samsun (Center, Alaçam, Bafra, Havza, Çarsamba, Ondokuzmayıs, Yakakent, Vezirköprü and Tekkeköy) in May and August 2003 from seedbeds and fields, respectively. Isolations from plants for *Rhizoctonia* samples were made as explained in Erper *et al*., (2002). The hyphal tips of *Rhizoctonia*-

like samples were transferred to Potato Dextrose Agar (PDA, Merck). For pre-identification, hyphal features and nuclear condition of the isolates were examined as explained in Karaca *et al*., 2002 after stained with Safranin O and 3% KOH (Bandoni, 1979). Binucleate isolates that exhibited *Rhizoctonia* form genus hyphal features (Sneh *et al*., 1991) were included in further analysis. Collected BN *Rhizoctonia* isolates were maintained on dried oats, using the method of Sneh *et al*., (1991).

Anastomosis group determination: Anastomosis group determinations of BN *Rhizoctonia* isolates were conducted according to the method of Karaca *et al*., (2002) and the relationships among the isolates were evaluated according to the anastomosis scale of Carling *et al*., (2002) and hyphal fusion frequency (FF) approach explained in Sneh *et al*., (1991). The tester isolates representing different AGs and ISGs of BN *Rhizoctonia* (AG-A to AG-S) used in AG determinations were provided by Prof. A. Ogoshi and Prof. E. Demirci.

DNA extraction: Mycelial powders used in genomic DNA isolations were prepared as explained in Gurkanli *et al*., (2009) and stored at -50˚C until DNA extraction. Genomic DNA was extracted using a Qiagen DNeasy® Plant Mini Kit, as specified by the manufacturer and stored at -20°C prior to use.

Polymerase chain reactions, DNA sequencing and data analysis: Primers, ITS4 and ITS5 (White *et al.,* 1990) were used for PCR amplifications of ITS-1/5.8S-rDNA/ITS-2 region. A 50µl PCR mixture was prepared using genomic DNA<1µg, 1.5 mM $MgCl_2$, 1.25U *Taq* polymerase (Promega, Go-Taq Flexi DNA Polymerase), 2.5 mM dNTP mix (Amresco), 10µl ×5 PCR buffer and 0.6 pmol (final conc.) of each primer. A MWG Primus thermal cycler was used for amplification in the following process: an initial denaturation at 94°C for 2 min., 30 cycles of 94°C for 40 sec, 55°C for 1 min., and 72°C for 50 sec; and a final period at 72°C for 5 min. The PCR products were electrophoresed on 0.8% agarose gel (Amresco, Solon, Ohio) in 1xTBE (Tris-Borate-EDTA) buffer, stained with Ethidium bromide and visualized with the GeneGenius Bio imaging system.

The rDNA-ITS regions of BN *Rhizoctonia* isolates were sequenced in both directions by Macrogen (Korea) with using ABI 3730 XL sequencer. In edition to the isolates collected in this study, we also sequenced the AG-A tester isolates, C517 and ED138 provied by Prof. A. Ogoshi and Prof. E. Demirci respectively. The sequences were assembled with using the computer software package SeqMan II module of the LASERGENE 99 system (Applied Biosystem) and aligned with CLUSTAL X (Thompson *et al.,* 1997) then optimized by hand. To present the phylogenetic relationships among isolates Neighbour-Joining (NJ) method was employed using computer package PHILIP v. 3.68 (Felsenstein, 2004). The bootstrap analysis of NJ trees were conducted with 1000 replications using the Seqboot and Consense tools of PHILIP. All our new sequences have been deposited in the EMBL database (accession numbers: FR734288-FR734303).

Pathogenicity determination: To determine the pathogenicity effects of BN *Rhizoctonia* isolates on tobacco seedlings, an *In-vivo* experiment was carried out as explained by Gurkanli *et al.,* (2009) and Ramamoorthy *et al.,* (2002). The results were evaluated according to 0-4 scale (Table 1) of Villajuan-Abgona *et al.,* (1996). The pathogenicity experiments were carried out with 12 replications for each isolate in Completely Randomized Design (CRD). Because the scale scores were categorical variable, they were analysed by Kruskal Wallis one way analysis of variance technique. Then non-parametric multiple comparison Dunn test (Siegel & Castellan, 1988) was performed for paired differences of isolates, (Significance level α=0.002).

Table 1. Origins, pathogenicity scores and EMBL database accession numbers of BNR-tobacco isolates and AG-A tester isolates.

Isolate	BNR-AG	EMBL database accession number	Origin	Pathogenicity scores	
				Mean rank	Median
T1	A	FR734288	Vezirköprü/Samsun	99,9[b]	2
T2	A	FR734289	Bafra/Samsun	65,8[ab]	0
T3	A	FR734290	Center/Samsun	80,3[ab]	0.5
T4	A	FR734291	Alaçam/Samsun	74,8[ab]	0.5
T5	A	FR734292	Ondokuzmayıs/Samsun	48,0[a]	0
T6	A	FR734293	Kavak/Samsun	152,5[c]	4
T7	Fb	FR734294	Çarşamba/Samsun	48,0[a]	0
T8	Fb	FR734295	Tekkeköy/Samsun	48,0[a]	0
T9	Fa	FR734296	Çarşamba/Samsun	48,0[a]	0
T10	Fb	FR734297	Tekkeköy/Samsun	48,0[a]	0
T11	A	FR734298	Havza/Samsun	149,8[c]	4
T12	A	FR734299	Vezirköprü/Samsun	147,0[c]	4
T13	A	FR734300	Kavak/Samsun	149,8[c]	4
T14	A	FR734301	Havza/Samsun	149,8[c]	4
(-) Control	-	-	-	48,00 [a]	0
C517	A	FR734302	Japan	-	-
ED138	A	FR734303	Erzurum/Turkey	-	-

Disease severity scores: 0= healthy plant, no lesions on the hypocotyl; 1=one or two lesions <0.25 mm long; 2. lesions <0.5 mm covering; 3=lesions >0.1 mm long and covering 10% to 100% of the hypocotyls; 4=seedling is dead. The letters above the mean ranks are indicating the pathogenicity groups constitute according to the pair-wise comparison of the pathogenicity scores.

Results

Sampling, identification and anastomosis group determination: Fourteen BN fungal samples which corresponded with the *Rhizoctonia* form genus criteria were isolated from tobacco fields and seedbeds in 9 different districts of Samsun. Anastomosis reactions between the BN *Rhizoctonia* tester isolates and tobacco isolates revealed three groups. The first group contains the isolates T1, T2, T3, T4, T6, T11, T12, T13, T14 whereas second group contains T7, T8, T9, T10 and the third contains T5. Isolates T2, T3, T4 of the first group anastomosed with each other and AG-A testers (ED138 and C517) with C1- category, where they did not anastomose with other testers. The other isolates of the first group (T1, T6, T11, T12, T13 and T14) anastomosed with each other and tester ED138 with C2 category and 34-42% FF, which indicates a moderate relation among these isolates. These isolates and ED138 also only contacted (C1-) with the tester C517. The isolates of second group (T7, T8, T9, and T10) contacted with AG-F tester (AH-6) with C1+ category whereas they did not anastomose with the other testers of BNR-tobacco isolates. On the other hand, isolates T7, T8, T10 anastomosed with each other with 54-68% FF whereas they just contacted (C1) with T9. Isolate T5 which was the only isolate in the third group did not anastomose with neither BNR testers nor the other BNR-tobacco isolates.

ITS-rDNA phylogeny: BNR isolates T1, T2, T3, T4, T5, T6, T11, T12, T13 and T14 were found as closely related to AG-A isolates from different localities and hosts (Fig. 1). In the NJ tree of AG-A, there were two major lineages supported with a bootstrap value of 52.4%. Our AG-A tobacco isolates were distributed in both of these lineages. Interestingly, isolates T1, T6, T11, T12, T13, T14 and ED138 which anastomosed each other with 34-42% FF and C517 (only contacted, C1-, with the other AG-A isolates)

clustered in the same monophyletic group (Lineage I), suggesting a more homogenous subset in AG-A, whereas isolates T2, T3, T4 and T5 consisted the second monophyletic group (Lineage II) in the tree. Although isolates in the second lineage found as phylogenetically closely related with each other, we could not find the same close relation in the anastomosis tests as we specified above, for instance isolate T5 in this lineage didn't anastomose with any other isolates. Martin, (2000) reported a similar result that his AG-A isolates from strawberry in California did not anastomose with some of the AG-A testers. We also could not determine an actual anastomosis (C3 or C2) between these two AG-A lineages. If we gather our results from anastomosis tests and molecular phylogeny with the finding of Martin, (2000), it is obvious that anastomosis tests may not be a reliable identification character for BNR AG-A and must be supported with molecular methods like rDNA-ITS sequence phylogeny. Our four BNR-tobacco isolates; T7, T8, T10 and T9 which anastomosed with AG-F tester were found as phylogenetically related with AG-Fb and AG-Fa subgroups respectively (Fig. 2). Although the bootstrap value at AG-Fa and -Fb dichotomy node is lower than 50% (35.6%), our AG-F NJ tree in Figure 2 is supporting the findings of Sharon *et al.,* (2007) and Sharon *et al.,* (2008) that the AG-F isolates may divide into subsets as AG-Fa and Fb in the respect of rDNA-ITS phylogeny. Our results from anastomosis tests are also supporting this suggestion.

Pathogenicity: As the result of pathogenicity tests, we determined three main groups (Table 1). First group includes the slightly virulent (T2, T3, T4) and non-pathogenic (T5) AG-A isolates as well as non-pathogenic AG-F (T7, T8, T9 and T10) isolates, whereas the second group contains the moderately virulent AG-A isolate (T1) and the third group contains the pathogenic (inducers of root rot of tobacco) AG-A isolates (T6, T11, T12, T13 and T14). Interestingly, our pathogenic and moderately virulent AG-A isolates grouped in Lineage I in NJ tree (Fig. 1), whereas non-pathogenic and slight virulent isolates placed in Lineage II. Therefore, these pathogenicity results are supporting the dichotomy in our AG-A NJ tree.

Discussion

In current study we isolated 14 BN *Rhizoctonia* samples from diseased tobacco seedlings and plants in Samsun province and identified 11 of them as AG-A, 1 as AG-Fa and 3 as AG-Fb at the result of anastomosis tests and rDNA-ITS phylogenies. Tobacco has been reported to be infected by *R. solani* isolates belonging to AG-1 through to -5 (Nicoletti *et al.*, 1999). Of these pathogens, *R. solani* AG-1, AG-2-1, AG-2-2, AG-4 isolates have been reported as inducers of non-foliar diseases (root rot, stem rot or sore shin and damping-off) where AG-3 isolates as inducers of foliar diseases (target spot) on tobacco (Shew & Melton, 1995; Gutierrez *et al.,* 1997; Nicoletti *et al.,* 1999). Although BN *Rhizoctonia* AG-A was reported as inducers of several diseases on different plants (Sneh *et al.,* 1991), it was not reported from tobacco so far, therefore according to available literatures this study contains the first report of BN *Rhizoctonia* AG-A as inducer of root rot of tobacco. Some molecular and conventional (anastomosis tests) analysis results from several studies (Martin, 2000; Sharon *et al.,* 2008) are suggesting that BN *Rhizoctonia* AG-A may not be a homogenous group and may contain several subsets inside. In this study we determined two different phylogenetic lineages of AG-A and these lineages were partly supported by the anastomosis reactions and pathogenicity tests. That is why our results provides important evidences for Sharon *et al.,* (2008)'s presumption for the existence of different subsets in AG-A. But it still needs further studies with using more AG-A samples isolated from a wide variety of host plants and different locations.

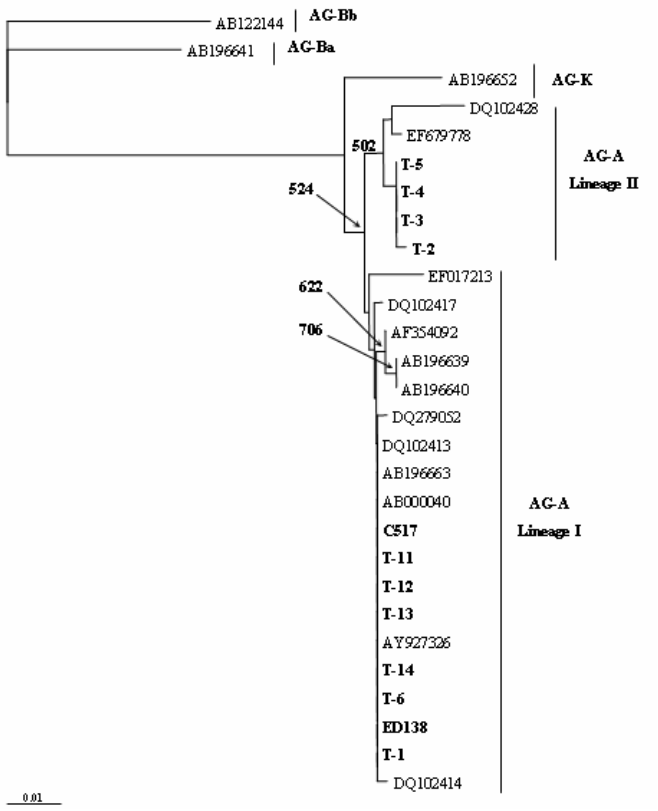

Fig. 1. Neighbour-Joining (NJ) tree showing the relations between AG-A isolates from tobacco and different hosts and also phylogenetically closest BNR-AGs (AG-K) to AG-A based on rDNA-ITS sequences. The tree based on F84 distances and rooted with AG-Ba and -Bb.

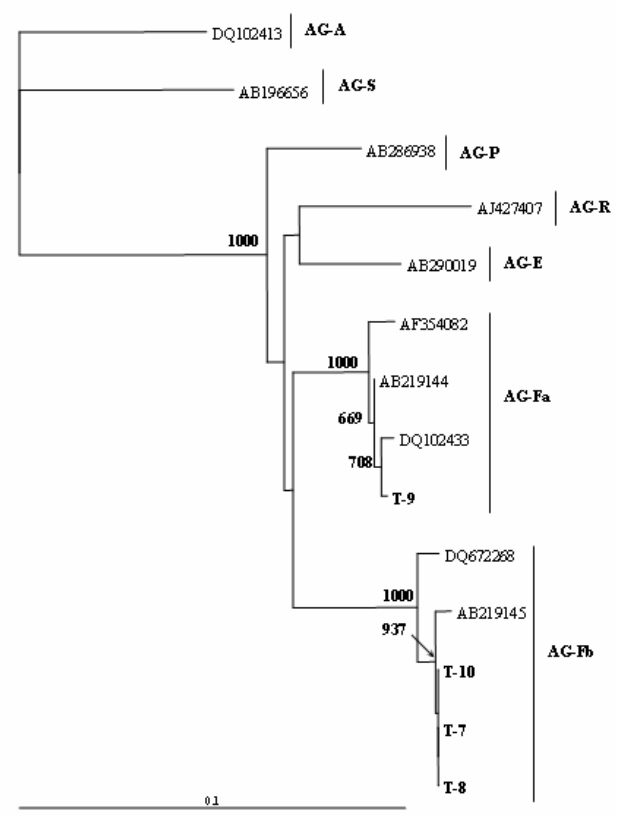

Fig. 2. Neighbour-Joining (NJ) tree showing the relations between AG-F isolates from tobacco and different hosts and also some phylogenetically closest BNR-AGs (AG-E, -P, -R, -S) to AG-F based on rDNA-ITS sequences. The tree based on Kimura F84 distances and rooted with AG-A.

BN *Rhizoctonia* AG-F was reported from several plants including bean, eggplant, onion, pepper and cabbage in Turkey by Demirci & Doken, (1995) and Eken & Demirci, (2004). But the identification of these isolates were made with anastomosis tests hence the subsets of the isolates were not apparent. That is why this study also contains the first reports of AG-Fa and Fb subsets from Turkey.

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