Research article

Biocontrol of Phytopathogen by *Pseudomonas fluorescens* R21, Isolated from Rice Rhizosphere in Thailand

PHRUEKSA LAWONGSA*
Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand
Email: phrueksa@gmail.com

NANTAKORN BOONKERD
Suranaree University of Technology, Nakhon Ratchasima, Thailand

FERGAL O'GARA
University College Cork, National University of Ireland, Cork, Ireland

NEUNG TEAUMROONG
Suranaree University of Technology, Nakhon Ratchasima, Thailand

Received 14 December 2011 Accepted 18 April 2012 (*: Corresponding Author)

Abstract Secondary metabolites produced by fluorescent *Pseudomonas* play key roles in the suppression of various soilborne plant pathogens. However, the performance of this biocontrol agent varies depending on the environment and host plant species. In this study, *In vitro* antagonistic activity against phytopathogens by *Pseudomonas fluorescens* R21, which was isolated from rice rhizosphere in Thailand, was investigated in comparison to *Pseudomonas fluorescens* F113, which was isolated from sugar beet rhizosphere in Ireland. The result of *in vitro* antagonistic activity showed that *Pythium* spp. was suppressed by strains R21 and F113. Then, strain R21 that has the ability to produce IAA and to control plant pathogen was investigated for *in vivo* antagonistic activity and also was screened for the production of secondary metabolites such as pyoluteorin, pyrrolnitrin, hydrogen cyanide and 2,4-diacetylphloroglucinol (DAPG). The result of *in vivo* antagonistic activity confirmed that *Pythium* spp. was suppressed by strains R21 and F113. In addition, the application of strain R21 to rice seeds significantly increased plant height, shoots dry weight and roots dry weight of rice while the application of strain F113 to rice seeds showed no significant difference when compared to control.

Keywords *Pseudomonas*, rice, *Pythium* spp.

INTRODUCTION

Agriculture over the past few decades is heavily dependent on the application of chemical inputs. However, many chemical pesticides are very toxic and thus result in contamination of environment. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant et al., 2005; Welbaum et al., 2004).

The introduction of *P. fluorescens* as a biocontrol agent offers a promising alternative to manage soilborne plant pathogens. However, the production of an antimicrobial compound varies among cultivars of the same species, and this has hampered the commercialization (Notz et al., 2001). The studies of the ability to produce antibiotic secondary metabolites and their plant growth promoting potential are important not only for understanding their ecological roles in the rhizosphere and their interaction with plants, but also for any biotechnological applications.

In this study, *P. fluorescens* R21, isolated from rice (*Oryza sativa*) rhizosphere in Thailand, that has the ability to produce indole-3-acetic acid (IAA) was selected (Lawongsa et al., 2008). Strain R21 was investigated for *in vitro* and *in vivo* antagonistic activity of pathogenic fungi and also screened for secondary metabolites production. These results will facilitate overcoming

©ISERD

existing limitations in the understanding of plant-microbe interactions of strain R21. In this work, *P. fluorescens* F113 isolated from sugar beet (*Beta vulgaris*) rhizosphere in Ireland was used as the reference strain to compare with strain R21.

MEHODOLOGY

Bacterial strains and culture conditions

Isolation of rice rhizosphere strain R21 was carried out by serial dilution. Nutrient agar and *Pseudomonas* isolation agar (Sigma-Aldrich) were used. Strain R21 was identified on the basis of growth characteristic, microscopy and biochemical tests. The strains of *Pseudomonas fluorescens* were maintained on Luria-Bertani (LB) agar at 4°C and grown at 28°C overnight, shaking at 150 rpm.

In vitro biological control assay

An inhibition of phytopathogen by the *Pseudomonas* strains on Potato dextrose agar (PDA) plates was performed as detailed in previous study (Lawongsa et al., 2008). Bacterial suspension of strains F113 and R21 was spotted 2 cm from the edge of the plate, and 0.1-0.3 cm square plug from a culture of *Pythium* spp. was placed at the center of the plate. The results were assessed after 3 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium.

Rice and soil preparation for the assay of *in vivo* antagonistic activity

The cultivated rice (*Oryza sativa* cultivar Pathum Thani 1) was obtained from Pathum Thani Rice Research Center, Pathum Thani, Thailand. The rice seeds were surface-sterilized with 70% ethanol for 1 min and shaken in 10% (w/v) NaOCl solution for 30 min. Seeds were then washed three times with sterilized distilled water by shaking (15 min each) (Prakamhang et al., 2009). Surface sterilized seeds were gnotobiotically germinated on sterilized wet tissue paper. After 3 days, rice seedlings in each treatment were transferred into pots (cm in diameter, 60 cm high) containing autoclaved soil. Three rice seeds were planted to a depth of 1 cm in each pot. Three replications for each treatment were done. Rice pots were placed in growth chambers maintained at 25 °C (± 1 °C) with 12 hours of light and 12 hours of darkness (Kato-Noguchi and Kanesawa, 2003).

Preparation of bacterial inocula and seed treatment for the assay of *in vivo* antagonistic activity

Cells of antagonistic bacteria for use in the assay of *in vivo* antagonistic activity were grown in King's Medium B broth (KMB) into a late exponential phase at 28°C with shaking at 150 rpm overnight. Cells were then harvest by centrifugation (5000 rpm/min for 5 min), washed twice and resuspended in 0.5% sterile NaCl solution. The rice seeds were soaked with bacterial suspension that was adjusted to about 10^8 colony forming units (CFU/ml) for each experiment and applied with *Pythium* spp. (1×10^6 spores/ml) (Timms-Wilson et al., 2000). Control treatments were inoculated in sterile distilled water and seedling with disease symptoms were recorded 4 weeks after planting. Plants were arranged in a randomized completed block design with three replications. Percentage of disease incidence was assessed 4 weeks after planting. Rice plants were sampled 4 weeks after planting to measure plant height, shoot and root dry weight.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at 5 % level using the statistical package of the social sciences (SPSS) version 17.0. The data presented are means standard deviation (SD) of three replicates.

©ISERD

Detection of pyoluteorin and pyrrolnitrin biosynthetic loci

Primers PltBf2 and PltBr were used for detection of the pyoluteorin biosynthetic locus of *pltB*. Primers PrnCf and PrnCr were used for detection of the pyrrolnitrin biosynthetic locus of *prn*C. PCR amplification was carried out in 25 µl reaction mixture containing 1× *Taq* DNA polymerase buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 1.5 mM MgCl2, and 0.06 units of Go*Taq* DNA polymerase (Promega). The PCR cycling program consisted of initial denaturation at 94°C for 2 min followed by 29 cycles of 94°C for 1 min, 58°C for 45 s, and 72°C for 1 min. The amplification products were electrophoresed in 1% agarose gels in 1× TAE buffer for 40 min at 100 Volt at room temperature, stained with ethidium bromide, and photographed under UV light (Mavrodi et al., 2001).

Detection of hydrogen cyanide production

The production of hydrogen cyanide (HCN) was measured as described by Castric, 1975. Whatman 3MM paper was soaked in a chloroform solution containing copper (II) ethyl acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylaniline) (5 mg/ml), and subsequently dried and stored in the dark. A piece of paper was placed in the lid of a Petri dish in which bacteria had been placed on SA agar. The Petri dish was incubated overnight at 28 °C. Production of HCN by the bacteria was indicated by blue coloration of the paper (Castric, 1975).

Detection of genes involved with DAPG production by PCR

Standard PCR mixtures and PCR conditions were employed: typically, an initial denaturation step at 96°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, then 30 s of primer annealing with temperatures dependent on the primer pairs used, and an extension of 1 kb per min at 72°C. DAPG genes were determined using *phlD* gene specific primers, Phl2a and Phl2b (Raaijmakers et al., 1997), and *phlA* gene specific primer, phlA-1f and PhlA-1r (Rezzonico et al., 2003).

RESULTS AND DISCUSSION

***In vitro* antagonistic activity**

The antagonistic activity from both strains R21 and F113 was summarized in Table 1.

Table 1 Measurement of diameter of fungal inhibition ring (cm) of *P. fluorescens* R21 against *Pythium* spp.

Pathogen	Diameter of fungal inhibition ring (cm)	
	R21	F113
Pythium spp.	1.4 ± 0.05	1.4 ± 0.05

The *in vitro* antagonism experiments with *Pythium* spp. (Figure 1) revealed that strain F113 showed the same level of antagonistic activity when compared to strain R21.

Assay of *in vivo* antagonistic activity

After two weeks, rice seedling inoculated with strains R21 and F113 showed damping-off symptoms. An initial symptom was developed as lesions on sheaths of lower leaves near the water line. After 4 weeks, the disease intensity in the treatment inoculated with *Pythium* spp. and strain R21 was 58.3 % while the disease intensity in strain F113 was 66.7 %. However, there is no significant difference in the disease intensity between applications of strain R21 and strain F113 against *Pythium* symptom (Fig. 2 and Table 2). These results indicated and confirmed the result of

©ISERD

in vitro antagonistic activity that the colonization of roots by *Pythium* spp. was suppressed by both strains R21 and F113. Additionally, there is no significant difference in plant height, shoots dry weight and roots dry weight of rice between applications of strain R21 and strain F113 against *Pythium* spp. Interestingly, the application of strain R21 to rice seeds showed significantly increased plant height, shoots dry weight and roots dry weight of rice when compared with control while the application of strain F113 to rice seeds showed no significant difference when compared to control.

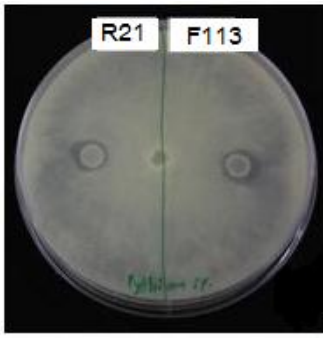

Fig. 1 *In vitro* antagonistic activity against *Pythium* spp. by strain R21 and strain F113

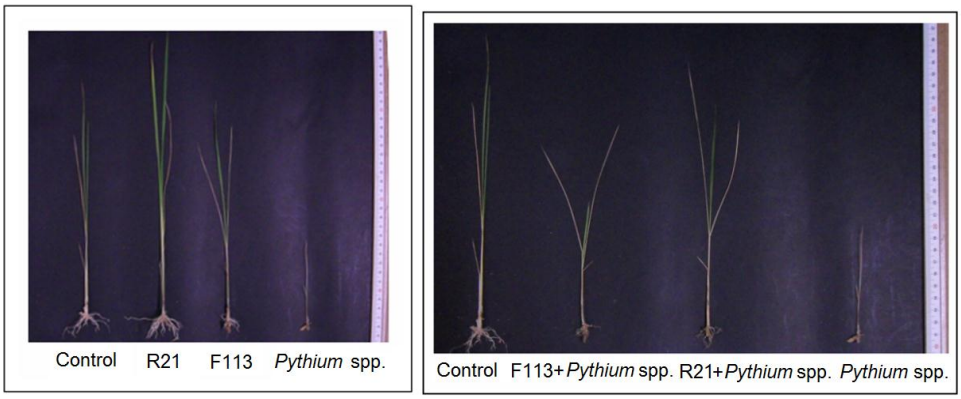

Fig. 2 *In vivo* antagonistic activity of strain R21 and strain F113 against *Pythium* spp.

Table 2 Disease incidence, plant height, and dry weight of shoots and roots of rice inoculated with strain R21 grown in the presence of *Pythium* spp.

Sample	% incidence	Plant height (cm)	Shoot dry mass (mg)	Root dry mass (mg)
Control	0^{c}	21.70 ± 1.57^{bc}	32.47 ± 2.50^{b}	11.44 ± 0.30^{b}
R21	0^{c}	28.77 ± 2.81^{b}	56.57 ± 6.14^{b}	22.22 ± 3.89^{b}
F113	0^{c}	22.40 ± 1.44^{b}	33.00 ± 7.05^{b}	10.15 ± 3.21^{b}
Pythium spp.	100 ± 0.00^{a}	9.40 ± 3.65^{d}	7.90 ± 0.98^{d}	2.17 ± 0.75^{c}
R21+*Pythium* spp.	58.3 ± 14.43^{b}	18.77 ± 2.85^{bc}	23.57 ± 5.95^{c}	5.56 ± 0.70^{c}
F1113+*Pythium* spp.	66.7 ± 14.43^{b}	17.67 ± 1.59^{c}	22.57 ± 1.05^{c}	5.82 ± 0.98^{c}
%CV	22.22	12.46	15.94	20.36

Means in columns followed by different letters are significantly different at 5 % level according to the Duncan's Multiple Range Test (DMRT)

Detection of secondary metabolites

Most biocontrol strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds. Pyoluteorin is composed of a resorcinol ring, derived through

©ISERD

polyketide biosynthesis (Nowak-Thompson et al., 1997). It is an antibiotic that inhibits oomycete fungi, including the plant pathogen *Pythium ultimum*, and suppresses plant diseases caused by this fungus (Howell and Stipanovic, 1980). The pyoluteorin biosynthetic gene cluster *pltLABCDEFG* is required for pyoluteorin biosynthesis (Nowak-Thompson et al., 1999). In this experiment, strain R21 was screened for the presence of pyoluteorin. *P. fluorescens* Pf-5 and CHA0 were used to be the positive control. The PCR result showed no PCR product was amplified from strain R21 with the PltBf2 and PltBr primers that amplified the predicted 773-bp fragment for detection of the pyoluteorin biosynthetic locus of *pltB* (Fig. 3). Pyrrolnitrin is a secondary metabolite derived from tryptophan and has strong antifungal activity (Zhou et al., 1992). DNA region confers the ability to produce pyrrolnitrin that contains four genes, *prnABCD*, each of which is required for pyrrolnitrin production. Pyrrolnitrin has been described as an inhibitor of fungal respiratory chains (Tripathi and Gottlieb, 1969) and synthetic analogues of pyrrolnitrin have been developed for use as agricultural fungicides (Ligon et al., 2000). In this experiment, strain R21 was screened for the presence of pyrrolnitrin. *P. fluorescens* Pf-5 and CHA0 were used to be the positive control. The PCR result showed no PCR product was amplified from strain R21 with the PrnCf and PrnCr primers that amplified the predicted 719-bp fragment of *prnC* (Fig. 3).

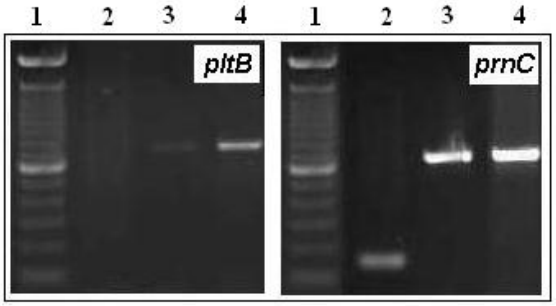

Fig. 3 PCR analysis of genes involved in pyoluteorin (*pltB*) and pyrrolnitrin (*prnC*) production. Lane 1, 100 bp ladder (Invitrogen); lane 2, R21; lane 3, Pf-5; lane 4, CHA0

HCN is a volatile compound which plays a role in biological control of some soilborne diseases (Haas and Defago, 2005). The cyanide ion derived from HCN is a potent inhibitor of many metalloenzymes, especially copper-containing cytochrome *c* oxidases (Blumer and Haas, 2000). In this experiment, strain R21 was screened for the presence of HCN. *P. fluorescens* Pf-5 and F113 were used to be the positive control for HCN detection. Strain R21 showed a negative result of hydrogen cyanide production (Fig. 4).

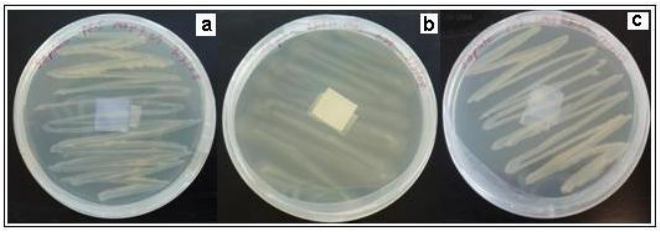

Fig. 4 Hydrogen cyanide production assay of strains Pf-5 (a), R21 (b) and F113 (c)

DAPG is the best-known phloroglucinol compound in a family of related molecules that includes MAPG and uncharacterized condensation products of DAPG and MAPG (Heeb et al., 2002). DAPG causes membrane damage to *Pythium* spp. and is particularly inhibitory to zoospores of this oomycete (de Souza et al., 2003). In this experiment, strain R21 was screened for the presence of DAPG. *P. fluorescens* Pf-5, CHA0, and F113 were used to be the positive control. The result showed that the positive amplification PCR product was revealed by strain R21 using

©ISERD

primers Phl2a and Phl2b that amplified the predicted 745-bp fragment of *phlD* (Fig. 5) and primers phlA-1f and phlA-1r that amplified the predicted 418-bp fragment (Fig. 5).

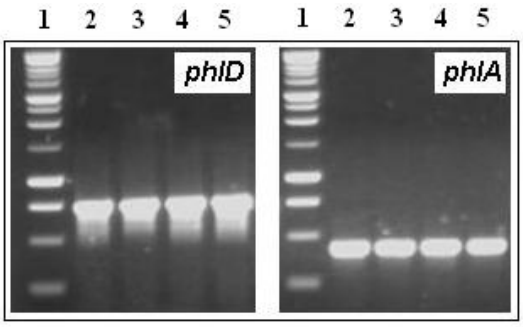

Fig. 5 PCR analysis of genes involved in DAPG production (*phlD* and *phlA*). Lane 1, 1 kb ladder (Promega); lane 2, R21; lane 3, Pf-5; lane 4, CHA0; lane 5, F113

CONCLUSION

Among biocontrol agents, *P. fluorescens* producing the polyketide antibiotic DAPG are important groups of plant growth promoting rhizobacteria (PGPR) that suppress root and seedling diseases on a variety of crops. In this study, the results of *in vitro* and *in vivo* antagonistic activity showed that *Pythium* spp. that caused damping-off and root rot was suppressed by strain R21 and F113. Interestingly, the application of strain R21 to rice seeds showed a significantly increased of plant height, shoots dry weight and roots dry weight of rice when compared with control while the application of strain F113 to rice seeds showed no significant difference when compared to control. This information indicated that the ability to colonize rice roots is variable between rhizobacteria, being these characteristics a reflection for their ability to compete for ecological niches in the rhizosphere. Moreover, the exertion an appropriate biological control of soil borne fungi relies on their ability to colonize roots efficiently; otherwise, their biocontrol character would be unusable. Thus, introduction of biocontrol agent to agriculture requires appropriate and compatible PGPR for the goal of making agriculture more sustainable. Moreover, an understanding of how biocontrol bacteria regulate the inhibition of pathogens is important for predicting the optimum environmental conditions of the bacteria to produce antagonistic compounds.

ACKNOWLEDGEMENT

This work was supported by a grant from Thailand Research Fund and Suranaree University of Technology.

REFERENCES

Blumer, C. and Haas, D. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol, 173, 170-177.

Castric, P. 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can. J. Microbiol, 21, 613-618.

Compant, S., Duffy, B., Nowak, J., Clement, C. and Barka, E.A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol, 71, 4951-4959.

De Souza, J.T., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V.and Raaijmakers, J. M. 2003. Effect of 2,4-diacetylphloroglucinol on *Pythium*: Cellular responses and variation in sensitivity among propagules and species. Phytopathol, 93, 966-975.

Haas, D. and Defago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Rev. Microbiol, 3, 307-319.

©ISERD

Heeb, S., Blumer, C. and Haas, D. 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J. Bacteriol, 184, 1046-1056.

Howell, C.R. and Stipanovic, R.D. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathol, 70, 712-715.

Kato-Noguchi, H. and Kanesawa, T. 2003. Growth promoting substance in rice root exudates. Environ. Control in Biol, 41, 377-380.

Lawongsa, P., Boonkerd, N., Wongkaew, S., O'Gara, F. and Teaumroong, N. 2008. Molecular and phenotypic characterization of potential plant growth-promoting *Pseudomonas* from rice and maize rhizospheres. World J. Microbiol. Biotechnol, 24, 1877-1884.

Ligon, J.M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann, D., Kempf, H.J. and Van Pee, K.H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pest Manag. Sci, 56, 688-695.

Mavrodi, O.V., McSpadden Gardener, B.B., Mavrodi, D.V., Bonsall, R.F., Weller, D.M.and Thomashow, L.S. 2001. Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. Phytopathol, 91, 35-43.

Notz, R., Maurhofer, M., Schnider-Keel, U., Duffy, B., Haas, D.and Defago, G. 2001. Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathol, 91, 873-881.

Nowak-Thompson, B., Gould, S.J. and Loper, J.E. 1997. Identification and sequence analysis of the genes encoding a polyketide synthase required for pyoluteorin biosynthesis in *Pseudomonas fluorescens* Pf-5. Gene, 204, 17-24.

Nowak-Thompson, B., Chaney, N., Wing, J.S., Gould, S.J. and Loper, J.E. 1999. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. J. Bacteriol, 181, 2166.

Prakamhang, J., Minamisawa, K., Teamtaisong, K., Boonkerd, N. and Teaumroong, N. (2009) The communities of endophytic diazotrophic bacteria in cultivated rice (*Oryza sativa* L.). Appl. Soil Ecol, 42, 141-149.

Raaijmakers, J.M., Weller, D.M. and Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol, 63, 881-887.

Rezzonico, F., Moenne-Loccoz, Y. and Defago, G. 2003. Effect of stress on the ability of a *phlA*-based quantitative competitive PCR assay to monitor biocontrol strain *Pseudomonas fluorescens* CHA0. Appl. Environ. Microbiol, 69, 686-690.

Timms-Wilson, T.M., Ellis, R.J., Renwick, A., Rhodes, D.J., Mavrodi, D.V., Weller, D.M., Thomashow, L.S. and Bailey, M.J. 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. Mol. Plant-Microbe Interact, 13, 1293-1300.

Tripathi, R.K. and Gottlieb, D. 1969. Mechanism of action of the antifungal antibiotic pyrrolnitrin. J. Bacteriol, 100, 310-318.

Welbaum, G., Sturz, A.V., Dong, Z. and Nowak, J. 2004. Fertilizing soil microorganisms to improve productivity of agroecosystems. Crit. Rev. Plant Sci, 23, 175-193.

Zhou, P., Mocek, U., Siesel, B. and Floss, H.G. 1992. Biosynthesis of pyrrolnitrin. Incorporation of ^{13}C, ^{15}N double-labelled D-and L-tryptophan. J. Basic Microbiol, 32, 209-214.

©ISERD