Diverse bacteria are known to colonize plants. However, only a small fraction of that diversity has been evaluated for their biopesticide potential. To date, the criteria for sampling and selection in such bioprospecting endeavors have not been systematically evaluated in terms of the relative amount of diversity they provide for analysis. The present study aimed to enhance the success of bioprospecting efforts by increasing the diversity while removing the genotypic redundancy often present in large collections of bacteria. We developed a multivariate sampling and marker-based selection strategy that significantly increase the diversity of bacteria recovered from plants. In doing so, we quantified the effects of varying sampling intensity, media composition, incubation conditions, plant species, and soil source on the diversity of recovered isolates. Subsequent sequencing and high-throughput phenotypic analyses of a small fraction of the collected isolates revealed that this approach led to the recovery of over a dozen rare and, to date, poorly characterized genera of plant-associated bacteria with significant biopesticide activities. Overall, the sampling and selection approach described led to an approximately 5-fold improvement in efficiency and the recovery of several novel strains of bacteria with significant biopesticide potential.

Keywords: ARDRA, biocontrol, microbial diversity, plant growth promotion

Biopesticides constitute a rapidly growing subset of agricultural biotech inputs because of their proven efficacy, return on investment for producers, and reduced risk to consumers and the environment. And while the organic and sustainable food markets have been a clear driver in the increased use of biopesticides to date, the discovery of novel active ingredients will be one key to further market expansion (Marrone, 2007) especially for commercialization models based on patent protection (Harman et al., 2010). Some plant-associated bacteria can suppress plant pathogens and promote plant health through the expression of various mechanisms (Kim et al., 2011). Because these mechanisms require contact and growth on plant surfaces, bioprospecting efforts aimed at developing microbial biopesticides have focused largely on screening microbial collections acquired from agricultural soils and plants.

Both soils and plants are known to be extremely rich reservoirs of microbial diversity, with estimates of the total diversity ranging from tens of thousands to over a million species world-wide though the exact number is practically impossible to calculate (Sloan et al., 2008). Despite this, just over one hundred species of microorganisms have been developed into microbial biopesticides, approximately one third of which are bacteria (Copping, 2004). And, of these, only about two dozen species representing just ten genera (Agrobacterium, Azospirillum, Bacillus, Brevibacillus, Burkholderia, Erwinia/Pantoea, Pasteuria, Pseudomonas, Streptomyces) have been commercialized as microbial biopesticides for the control of plant diseases. Several more genera (Chromobacterium, Chrysobacterium, Klebsiella, Lyso bacter, Mitsuria, Paenibacillus) contain strains that are currently under development as microbial inoculants. More bacterial genera have been anecdotally reported to be associated with plants, but their potential to affect plant health remains largely uncharacterized. And, while isolates of a dozen other genera have been reported to display biological disease control or plant-growth promoting abilities (Rodríguez-Dias et al., 2008), most of these are still poorly characterized with relatively few strains assayed for biopesticide potential. Thus, there remains a great opportunity to characterize and develop novel plant-associated bacteria as biopesticide agents. The question is how, given limited
resources, might one most efficiently access that diversity and focus R&D efforts on new and different agents?

The traditional approach to developing microbial inoculants for plant health disease suppression has been to first isolate large collections of microorganisms then systematically screen them for activity; first using simple and cheap assays, then more complicated and expensive bioassays to identify those isolates with the greatest activity (Favel, 2005). More recently, molecular techniques have provided a glimpse of a much richer diversity of plant-associated microbial communities (Fierer et al., 2006; Yang et al., 2001). While much of this diversity remains uncultured in the laboratory, accessing novel phylogenetic groups through simple modifications of standard culture methods can be readily achieved (Nunes de Rocha et al., 2009; Qin et al., 2009). Molecular techniques have been combined with the culture-based techniques to identify and recover of novel species and subspecies of microorganisms with biopesticide activities (Borneman and Becker, 2007; Benitez and McSpadden Gardener, 2009). And while this newer approach to “identify then isolate” novel microorganisms with biopesticide potential is now being implemented with success in several laboratories, it is heavily dependent on sophisticated technical and statistical analyses of microbial community structure. Because of this, we reconsidered how the more traditional approach of “isolate then identify” might be systematically refined to increase the success rate of discovering truly novel microbial agents with biopesticide potential.

**Materials and Methods**

**Media and storage conditions.** Isolates were cultivated on solid and liquid media of four different recipes in this study. 1/3 × King’s Media B medium (KB) consisted of proteose peptone 6.7 g (Bacto, USA) monopotassium phosphate 0.4 g (Sigma Life Sciences Inc., USA), magnesium sulfate heptahydrate (Sigma life science Inc.) and 3.3 ml of glycerol (Junsei chemical Inc., Japan) per liter. 1/10 × tryptic soya medium (TS) was made with 3 g of Tryptic soil broth (Difco, USA) per liter. 1/10 × Tryptic Soya Agar (TSA; top) and 1/3 × King’s Media B Agar (KBA) or inoculated into liquid media of the same composition. For subcollection 1B, the washes were serially diluted 3.5 times × 6 dilutions in distilled water and either plated directly onto TSA or KBA or inoculated into liquid media of the same composition. For 24 hrs, 20 micro liters of the liquid cultures were plated from each dilution onto the corresponding solid media. After 2 days of incubation on solid media, individual colonies were picked and stored for further analysis.

For the 1st collection, representing a mixture of 24 samples from 16 different species, approximately 1 g of material was recovered from either the roots or leaves of plants grown on the research farm, arboretum, or landscape of the Chonnam National University campus on Oct 6, 2009. Individual plant samples were classified to genus, and, where possible species. Samples were placed in 10 ml of sterile water and incubated overnight at room temperature. Samples were then vortexed, and, for subcollection 1A plated directly onto 1/10 × Tryptic Soya Agar (TSA) or 1/3 × King’s Media B Agar (KBA) or inoculated into liquid media of the same composition. For subcollection 1B, the washes were serially diluted 3.5 times × 6 dilutions in distilled water and either plated directly onto TSA or KBA or inoculated into liquid media of the same composition. For 24 hrs, 20 micro liters of the liquid cultures were plated from each dilution onto the corresponding solid media. After 2 days of incubation on solid media, individual colonies were picked and stored for further analysis.

For the 2nd collection, representing the phyllosphere, individual leaves were sampled from the landscape around Agriculture Building IV on the campus of Chonnam National University (Gwangju, Korea) on Oct 10, 2009. Individual samples from different plants that were readily classified to genus. Individual leaves were pressed onto 1/10 × Tryptic Soya Agar (TSA; top) and 1/3 × King’s Media B Agar (KBA; bottom). They were then dropped into a 50 ml tube containing 15 ml of sterile water, briefly vortexed and left to incubate at room temperature in the dark. After 12 hr and 120 hr, the tubes were vortexed for 60 sec, and 20 microliters of each wash were plated onto TSA or KBA and incubated for 2 days prior to colony picking.

For the 3rd collection, representing the rhizosphere, soils were collected from around Korea between November 16 and December 14, 2009. With the exception of sample 1 which was from a rice field, all other soils were taken from vinyl houses where various vegetables had been grown and harvested approximately one month prior to sampling. Approximately 3 kg of soil from the top 20 cm was mixed in situ at each location prior to being bagged and shipped to Gwangju. Soils were stored at room temperature in the dark prior to analyses. Soil chemical analyses were conducted by Jeonnam Agricultural Research and Extension Services