Insights into the Biology, Diversity, and Origins of Weedy Red Rice and the Use of Phylogeographical Structures to Control its Seed-mediated Contamination in Taiwan

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ABSTRACT

Weedy red rice (WRR) possesses traits, including seed dormancy and shattering, that facilitate its infestation in rice paddies from one crop season to the next. These plants are not only the potential source of pollen-mediated gene flow and hosts for diseases or other pests, but also are competitors for fertilizer due to their vigorous growth. In addition to increased production costs to control this weed, the red pericarp and undesirable eating qualities of WRR lead to reduced product value, consequently putting production constraints on the global rice industry. In rice cultivation regions world-wide, the rice industry is in search of effective ways to control WRR. The rice production system in Taiwan relies on transplanting which is recommended for effective control of weeds in rice fields during the seedling stage. However, the infestation of WRR in rice paddies has become increasingly severe in the past few years in Taiwan. Although WRR occurs at a rate of only 0.5 to 1% in contaminated paddies, it can be spread easily through use of shared field equipment among rice fields. Ratoon cropping or tillage immediately after harvest increases the population densities of WRR, resulting in future yield losses. Effective control strategies for WRR should meet the balance between economic benefit, efficiency, and feasibility. The recommended WRR control measures under a transplanting system begin with irrigating the paddy field after harvest to induce the sprouting of shattered seed, followed by plowing the WRR seedlings into the land. Herbicide is then

applied three times in succession every seven days to kill emerging WRR and to field infestations. The final step is the manual removal of the remaining off-type plants of WRR.

Keywords: weedy red rice, seed shattering, seed dormancy, transplanting system, control strategy

INTRODUCTION

Weeds, soil fertility, availability of fertilizers, and variable climates are the major factors that limit rice growth. Weedy red rice (WRR) and cultivated rice are classified under the same genus and species (*Oryza sativa* L.) and have similar appearance and features; moreover, WRR plants in Taiwan are typically short, making them difficult to distinguish from crop rice, and exhibit rapid growth and high levels of seed shattering and dormancy, all of which are traits that make them invasive weeds. Consequently, once WRR forms a stable population in cultivated fields, controlling it and tracking its distribution patterns become difficult [(29)]. The hazards that WRR causes are detailed as follows: (1) Because of high levels of seed shattering and dormancy, seeds become established readily in the soil seed bank, resulting in plants that become a medium for gene flow as well as pest and disease transmission; (2) The biomass growth advantage of WRR from seedling to maturity leads to nutrient competition; (3) WRR exhibits negative qualities such as a red pericarp and undesirable cooked texture. All of these hazards lead to a sharp rise in rice production costs and decline in the commercial value of cultivated rice, hindering the development of the global rice industry. Hence, governments worldwide have actively searched for solutions to the hazards of WRR [(4)].

In addition, WRR has been an obstacle in the development of rice industries worldwide [(3, 16, 29)]. The invasion of WRR has been particularly problematic in Brazil, South Korea, and the United States of America (USA), where various WRR biotypes are present and rice planting is generally mechanized [(9, 15, 25)]. WRR has reduced rice production in some countries by 10%–50% and prevented subsequent rice planting in some cultivated fields; a density of 35–40 WRR plants per m^2 hampers the production of tall varieties of rice by 60% and that of dwarf varieties by as much as 90% [(22)]. In Japan, where a transplant system is implemented, a WRR density of 5 WRR plants per m^2 reduces rice production by 10% [(20)]. Furthermore, reduced rice production caused by 1–3 WWR plants is equivalent to that of 5–10 plants of barnyardgrass (*Echinochloa crus-galli*). WRR outweighs barnyardgrass in its damage to rice production and is difficult to contain because its appearance is so similar to that of cultivated rice [(21)].

To effectively prevent the invasion, latency, and spread of WRR as well as reinforce the knowledge of rice farmers regarding its detriment to rice industries, this study described the morphological diversity and phylogeographical structures of WRR, and generated control strategies for farmers to reference.

High Morphological Diversity and Adaptability of WRR

WRR is notorious for its high morphological diversity and environmental adaptability. In the *Japonica* rice production zones in Italy, WRR is divided into three variants, namely the long-awned, short-awned, and awnless strains. Long-awned WRR has the longest flag leaves and grains of the three variants; short-awned WRR produces small seeds and exhibits low germination rates; and awnless WRR has wide grains and high germination rates after 30 days of maturity. In particular, long-awned WRR displays the highest morphological diversity and environmental adaptability of all three variants (8). The WRR in the southern USA has two major variants, namely the strawhull awnless and blackhull awned strains. Strawhull awnless WRR features earlier heading dates than those of cultivated rice, whereas blackhull awnless WRR exhibits later heading dates than those of cultivated rice; both strains have a greater plant height than cultivated rice. In addition to hull color and awn length diversity, the characteristics of the WRR plants vary between strains. The WRR plants having a dense plant architecture and long awns have been noted for their high germination rates and seed vitality, whereas those with a loose plant architecture and no awns were noted for high tolerance to salt (23). In the *Indica* rice production zones in Thailand, the early generations of WRR were categorized as a mixture between the cultivated crop and weedy biotypes according to the WRR morphology inspection in multiple regions in 2005–2009 (27). This may have been caused by crossbreeding between the two biotypes (17, 18); following the accumulation of invading generations, WRR gradually became identical to cultivated *Indica* rice in appearance. Nevertheless, regardless of its generations and regions, WRR exhibits high adaptability to various cultivation methods employed in rice fields because of its early maturity, high levels of seed shattering, and robust productivity (27). In 2016 Cheng et al. (4) compared the morphological diversity of 521 locally collected WRR strains with that of *Indica* and *Japonica* rice and previously obtained red rice germplasm collections. The results indicated that all 521 WRR plants exhibited earlier maturity, greater seed shattering, more tillers, and shorter plant heights than cultivated rice plants; furthermore, pericarps were consistently red (Fig. 1). In a follow-up principal component analysis, the WRR populations exhibited similar degrees of dispersion with those of the cultivated rice; their geographic distributions were similar, but the grains of the WRR populations were identical in appearance to the wide and short grains of the early *Indica* crop rice varieties, and profoundly different from the slender grains of recent *Indica* varieties and the short and round grains of *Japonica* varieties. It is speculated that the grain traits of off-type plants were not influenced by human selection when these plants were eradicated intensively from rice nurseries. This indicated that the WRR collections are closely associated with the early rice strains.

Divided Arguments Worldwide on the Origins and Evolution Mechanisms of WRR

The genetic backgrounds of WRR vary considerably between countries, and arguments regarding its origins remain divided among scientific disciplines; however, the various hypotheses pertaining to the evolutionary methods of WRR have been supported by different studies [(7)]. According to De-Wet and Harlan [(6)], WRR originated from the adaptation of wild rice to agricultural habitats. In environments where no native wild rice biotypes are present, these can be introduced as a result of seed movement through trade and other means. For example, the WRR in Brazil is hypothesized to originate from Africa during the colonial era [(2)]. WRR is often closely associated with the genetic background of the rice varieties commonly produced in the areas to which it has been introduced. In Thailand and Malaysia, where rice can be planted every season and the growth areas and heading dates of cultivated rice overlap with those of its wild relatives, the weedy traits of wild rice have been introduced as a result of outcrossing with cultivars [(5, 26)]; subsequently these descendants evolved into WRR. Moreover, gene flow between wild and cultivated rice has also been identified in the USA [(13)]. In areas without wild rice, cultivated rice evolves into WRR after the adaptive mutation and de-domestication of the cultivated species and the accumulation of multiple effective mutations [(10, 12, 14, 19)]. In Brazil, China, South Korea, and the southern USA, WRR apparently originated from a background of closely related *Indica* rice [(12, 15, 19, 25)], whereas in Italy and California, WRR is speculated to have originated from crossbreeding among the *Japonica* species [(8, 29)].

According to the whole genome sequencing and domestication-related gene analyses employed by Li *et al.* [(12)], the WRR in the USA was produced from the de-domestication of cultivated rice and that weedy traits are sustained through selection in a few areas of the genome of the species. Qiu *et al.* [(19)] compared the de-domesticated genome sections of WRR with the domesticated genome sections of cultivated rice, revealing that the de-domestication of WRR did not involve changing the genotypes of the cultivated species back into wild genotypes, but it did involve adopting new genetic variations and molecular mechanisms to adapt to changing environments. On the basis of allele frequency distribution, a significant difference was identified among *Indica* and *Japonica* WRR groups in their standing variations and new mutations. Genetic diversity of WRR has been augmented through balancing selections to adapt to complicated survival environments, which is the opposite of the forward selections of cultivated rice. These results enhance existing knowledge of the genetic domestication and de-domestication mechanisms of cultivated crops, enabling an understanding of the environmental adaptation mechanisms of WRR and an exploration of strategies to prevent and eliminate it. However, the origins of WRR vary with its background cultivation ecology. In Taiwan, WRR has become adapted to the transplantation system, which has exacerbated its threat to the rice industry of Taiwan.

Historical Significance, Immediate Causes, and Ultimate Causes of the WRR in Taiwan

In Taiwan's pre-twentieth century agricultural era, cultivated rice primarily consisted of environmentally competitive tall *Indica* cultivars owing to insufficient technology, facilities, and resources. At that time, contamination of cultivated rice with red, black, and glutinous rice landraces, as well as with barnyardgrass, was severe and undermined the quality and appearance of rice products. During 1906–1921, systematic WRR control strategies were conducted to effectively contain the contamination. In 1953, a general survey of the *Indica* rice cultivation area revealed dwarf blackhull long-grain rice as the leading local *Indica* landrace. However, *Indica* red rice, which is drought- and salt-tolerant, was also cultivated by farmers after the primary crop had been harvested in the same year or on arid land; a total of 10 local land races of *Indica* red rice suitable for upland cultivation were recorded to have been planted. In 1959, the local red rice land races became extinct when the highly productive semidwarf "Taichung Native 1" *Indica* variety entered mass production and irrigation infrastructure was improved. Thus, more than 50 years of red rice cultivation was recorded in the rice cultivation history of Taiwan [(11)].

According to a 2005 analysis of rice seed nurseries, most off-type plants in Taiwan were tall, had early maturity, and slender, long grains [(28)]. Further examination revealed that these off-type plants originated from natural crossbreeding, contamination, or unsatisfactory uniformity among the contemporary varieties. Particularly, the samples displayed insufficient genetic similarities with their neighboring cultivated plants; these samples exhibited large numbers of tillers (36.4), average dwarf height of 73.2 cm, early maturity, and intermediate size grains. Their genetic backgrounds were highly homozygous and were similar to the *Indica* background, rendering the sample plants identical to the recent biotypes of WRR that predominate [4].

The rice production and sales policy has recently changed from recommended fallowing (1997) to a system where farmers are subsidized for cultivating leased fallowed lands (2012). Starting from 2003, the Environmental Protection Agency in Taiwan prohibited burning straw to reduce the detrimental effects of high temperature on soil surfaces. However, low-cost production methods such as ratoon stubble and volunteer rice have been applied in some regions during the second cultivation period. After 2012, severe red rice contamination was identified during the second cultivation period even though high-quality rice seed was planted during the previous cultivation period, leading to a drop in the government procurement price for paddy rice. In 2014, rice seed nurseries were invaded by WRR; 5% of registered seed fields (six fields) and 24% of certified seed fields (137 fields) were contaminated with red rice at an average rate of 0.47 ± 0.55%. In 2015, red rice contamination was detected in approximately 43% of the 187 townships in Western Taiwan where paddy rice was harvested for public stocks (data not published). Starting from the second cultivation period in 2014, Taiwan authorities discontinued the procurement of rice produced through perennial roots or seed shattering; however, these low-cost rice production approaches have continued and are a persistent source for the spread of WRR.

Phylogeographical Structures of Weedy Rice

Weedy rice can produce a large number of seeds at maturity and these can be accidentally mixed with cultivated rice seeds during harvest, which may promote the long-distance dispersal of weedy rice seeds due to shipment of seedlings between regions. In Taiwan, farmers grow rice based on a transplanting system, and generally use agricultural machinery during soil preparation, transplanting, fertilizer application and harvesting. The transplanting model is helpful in the control of weeds, and the purchase of seedlings from specialized commercial nurseries should reduce the occurrence of volunteer seedlings. In the field survey of 2015, we collected rice samples from seven counties and found that the contamination of weedy red rice was serious in 83 townships (Fig. 2). The contamination rate of each township is the average of all paddy fields in the same area. Of the four townships in Taoyuan City, Daxi District had the highest contamination rate of 0.73%, and neighboring Pingzhen, Longtan, and Myrica rubra District possessed red rice contamination rates of 0.17-0.41%. Among the five townships in Miaoli County, contamination rates were highest in Houlong Town, up to 0.51%, and decreased sequentially in the neighboring towns of West Lake, Tofen, Tongxiao, and Yuanli Town, ranging from 0.15 to 0.40%. Similar cases have occurred in Taichung, Changhua, Yunlin, Chiayi and Tainan counties. The contamination rate exceeded 0.6% in Daxi District of Taoyuan City (0.73%), Huatan Township of Changhua County (0.69%), Nantou City of Nantou County (0.74%) and Dongpotential Town of Yunlin County (0.85%). It is worth mentioning that more than half of the eight townships had a contamination rate of 0.20-0.40%, indicating that the invasion of weed type red rice is widespread and serious.

In order to establish an effective control strategy for weedy red rice, the first priority was to identify the degree to which the invasion in Taiwan occurred through seed-mediated contamination or pollen-mediated contamination, and if the transport distances in these two possibilities were significantly different from each other. In Sri Lanka for example, the abundant within-population genetic diversity coupled with limited population genetic structure and differentiation was likely caused by considerable seed-mediated gene flow of weedy rice along with long-distance exchange of farmer-saved rice seeds between weedy-rice contaminated regions (10). Knowledge of genetic diversity and spatial structure of weedy rice populations will facilitate the design of effective methods to control this weed by tracing its origins and dispersal patterns in a given region. In our study, we used information on genetic diversity and geographical location to understand the transmission patterns of Taiwan's weedy red rice. First, we used molecular markers to determine the genetic clusters of weedy rice populations and then measured the level of genetic diversity among different sub-populations. Second, we plotted the genetic cluster information of weedy rice on a map to reveal the geographical distribution of each group.

WRR Control Strategies Adopted in Japan

The WRR control strategies adopted by Japan, which uses a transplantation system similar to Taiwan, can be applied to treat WRR in Taiwan. These strategies have reduced the WRR contamination rate in fields with a WRR density of 100 plants/ 0.1 ha from 2.3% to 0.1%, fulfilling the safe production standard (20). These cultural practices include submerging fields with shallow water and plowing before planting, applying a three-phase herbicide treatment, or removing off-type plants manually, which are used on 78%, 20%, and 2%, respectively, of the areas treated for WRR. (20). Although various strategies are applied to eradicate WRR, a minimum rate of 99.6% depletion is required for lowering the contamination rate annually; a 95% depletion rate causes the contamination rate to rise from 5% to 15% in three years (24).

The focal points of WRR control during each cultivation period as used in Japan are listed as follows (20):

1. Rice variety purity control and management: Only rice varieties qualified through examination are permitted for use; using self-reserved seed or seedlings of questionable purity raises the risks of contamination.
2. Cultivated seed and rice nursery management: When broken grains and by-products of rice milling are used in the tray medium for growing seedlings, they must decay prior to use. Alternatively, rice mills should enforce the elimination of any residual germs within the pericarps. Use of any seed from contaminated fields is forbidden.
3. Pre-cultivation plow management:
   A. Plowing residual rice plants is avoided. The germination periods of WRR are closely related to its dormancy, maturity, and buried depths. WRR plants that are buried at a depth of 1 cm germinate at day 10 and reach a germination rate of 80% at day 14. Those that are buried at a depth of 3 cm germinate at day 14 and reach a germination rate of 70% at day 40. Those buried at 5 cm display no signs of germination even at day 35. These reports explain the factors leading to the emergence of volunteer plants in cultivated fields. Accordingly, plowing should be avoided before any control strategies are applied to prevent seeds from entering the deep layers of soil.
   B. When crop rotation is applied, gramineous selective herbicides should be used alternatively. The dormancy of residual seeds in the deep layers of soil are disrupted and any residual plants are eliminated when plowing. Seed at the soil surface or during fallowing periods may be consumed by birds, lowering germination rates.
   C. A long-period of flooding should be applied before preparing the soil. The finely prepared soil is covered with a shallow flood for a minimum of 30 days before plowing or from the previous cultivation period to reduce the WRR survival rate to

50%.

D. The soil should be managed after each rice harvest. Furthermore, the flooding treatment should be applied before preliminary plowing. One week after the germination begins during the treatment, at which point the sheath has grown to 20 mm, plowing is performed once to lower the germ survival rate by 10%. Nonselective herbicides are the most effective when used before plowing, raising the WRR elimination rate in a field with a contamination density of 12.7 plants per m^2 to as high as 78% when the soil is plowed after germination.

E. The soil should be cleaned thoroughly using high-temperature steam when environmentally friendly cultivation approaches are applied. A self-propelled high-temperature fumigation machine should be used to clean the cultivated fields, thereby eliminating seed in the shallow soil layers and killing the volunteers.

4. Field management after transplantation: A three-phase herbicide treatment is implemented. Pre-germination and post-germination herbicides are applied to the WRR seeds at various depths of soil, once during transplantation, followed by additional applications at seven and 14 days after transplantation. The herbicides butachlor and pretilachlor can be applied three times to effectively eliminate volunteer plants and improve the WRR elimination rate in a field with a contamination density of 12.7 plants per m^2 by 20%. Attention must be paid to the quality of herbicides, time points of their implementation, and maintenance of flooded fields, all of which affect the effectiveness of the herbicide. Moreover, the growth status of WRR must be considered. Selective herbicides are only effective against volunteer plants before or immediately after their germination, but not against seedlings having one or two leaves emerged.

5. Field management after heading:

   A. Off-type plants must be thoroughly uprooted and removed from cultivated fields.

   B. WRR typically exhibits earlier heading dates and higher morphological variation than cultivated rice does. Therefore, the plants that display inconsistent heading dates and plant traits compared with surrounding plants must be removed.

   C. Off-type plants must be uprooted within 14 days after the heading date. The maturity rate of shattered seeds increases from 10% at 14 days after heading to 50%–70% at 21 days after heading.

6. Sequence of harvest: Harvesters must be thoroughly cleaned after use; moreover,

harvesting crops in less contaminated fields must be prioritized over harvesting those in more contaminated fields.

CONCLUSION

WRR exhibits short growth periods and begins its seed shattering early and at high levels. Although soil preparation is performed before transplantation, a high number of residual seeds remain in the soil; the burial depths of these seeds increase through plowing, delaying and prolonging the growth of WRR plants after transplantation. Therefore, flooding must be implemented to thoroughly remove shattered seeds before plowing. Subsequently, selective herbicides should be applied once every seven days for a total of three times to ensure the delay of volunteer plant growth by a minimum of 21 days, which prevents WRR from entering its heading dates by the harvest period and breaking its seed production. Finally, the WRR plants and any other volunteer rice plants surrounding cultivated fields must be removed manually and comprehensively. Systematically understanding the weedy traits and genetic origins of WRR is critical to eliminate it economically and effectively, mitigating its effect on the development of rice industries.

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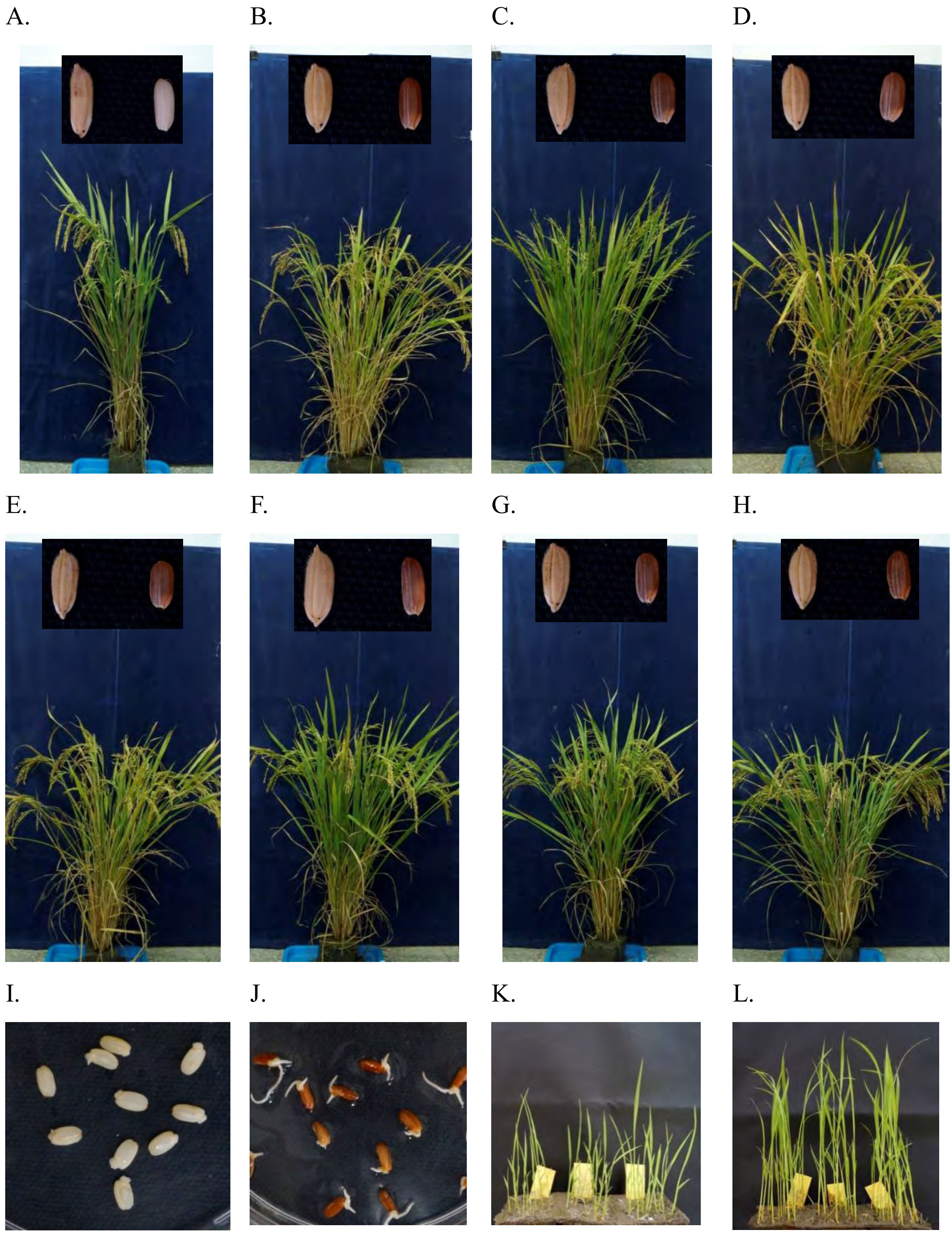

Fig. 1. Morphology, germination and seedling growth of cultivar and weedy red rice. The plant type and grain of "Taichung sen waxy 2" was different compared with the weedy rice accessions (B-H). The check variety "Tainan 11" (I), which was soaked for 3 days obviously germinated slower than weedy rice (J). The seedling growth of the weedy red rice (L) was greater than that of the control "Tainan 11" (K) after 16 days of soaking.

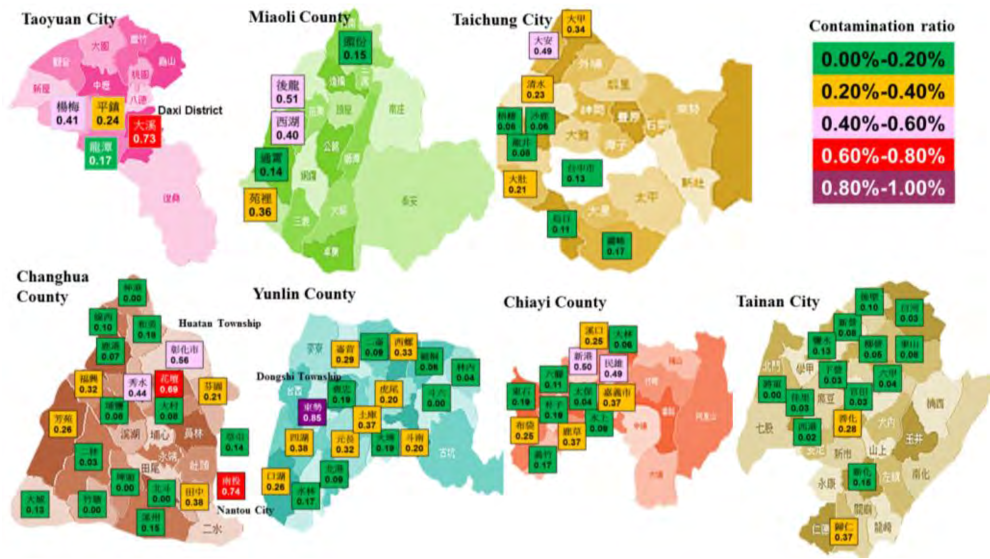

Fig. 2. The contamination ratio of weedy red rice was serious in 83 townships from 7 counties in the field survey of 2015. The contamination rate of each township is the average of all paddy fields in the same area.