Stabilisation of promising breeding rice plants resistant to *Pyricularia oryzae*

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Abstract

The aim of the research was to create stable breeding material of rice (*Oryza sativa* L.) resistant to rice blast (*Pyricularia oryzae*) using the biotechnological method of anther culture *in vitro*. Anthers from 20 perspective *F₂* and *F₃* hybrids of carriers of different genes of resistance to rice blast were used. The phytopathological assessment of the rice doubled haploid lines resistance to *Pyricularia oryzae* was performed in field experiments. Correlation of resistance level to quantitative signs of productivity of the received rice lines was assessed using the statistical analysis. A high sensitivity of all 20 rice genotypes to *in vitro* conditions was found. The level of callus formation ranged between 3.4% and 82.5% with the average of 23.7%. The level of green plants regeneration from obtained callus varied between 0.16% and 13.3% with the average of 3.3%. The level of albino plants regeneration was 0.6%. On average, approximately 35% of regenerated plants obtained in the culture were able to produce seeds indicating that doubled haploid lines were obtained. Most of them were characterised by a high plant productivity: 21.80–60.42 g compared to 10.31 g for the productivity of standard cultivar ‘Vikont’. A higher productivity was explained by a higher rate of productive bushiness. Eight lines showed a high level of resistance to rice blast (9 points), and 18 lines had an intermediate level of resistance.

Keywords: rice, androgenesis *in vitro*, regeneration, piriculariosis, doubled haploid.

Introduction

The rice (*Oryza sativa* L.) production accounts for 8% of the overall grain production of cereal crops. In Ukraine, only cultivars of white rice belonging to the Indica rice subspecies (round-grain rice) are grown. The most harmful disease for this plant is rice blast (*Pyricularia oryzae*), which annually causes significant losses in all rice-growing regions. The shortage of the harvest can reach 15–40%, and in years of epihytosis, rice blast can reduce the yield by 50% (Wilson, Talbot, 2009). Consequently, one of the main tasks of rice breeding is the creation of high-yielding cultivars of rice resistant to this disease.

Ukrainian scientists concentrate their efforts on the scientific research aimed at creating the breeding material resistant to the causative agent of rice blast by combining Pi-ta and Pi-b resistance genes in one genotype. This research is being done with the involvement of modern biotechnological approaches, namely androgenesis *in vitro* (the creation of the stable rice lines in anther culture) followed by DNA typing of plants (the breeding of rice regenerants with Pi-b and Pi-ta resistance genes) (Shpak, 2015).

Anther culture *in vitro* is widely and successfully used in selection programmes of rice-sowing countries of the world (Murovec, Bohanec, 2012; Tripathy et al., 2019a; b; Samantaray et al., 2021; Savenko et al., 2021; Upadhyay, 2022). In Ukraine, the biotechnological research for obtaining rice double haploids *in vitro* has been conducted since 2011. Based on previous studies (Shestopal et al., 2015; 2020; Zambriborshch et al.,...
the methodological recommendations describing the improved biotechnology of obtaining androgenic double haploids of cultivated rice were developed. The technology is based on the combination of methodical approaches: the optimal pre-treatment of spike under conditions of a low positive temperature and the cultivation of anthers and further formed callus on the improved nutrient media. The proposed technology increased the regeneration frequency by 1.5 times (Zambriborsch et al., 2016).

The main purpose of the research was the creation of a stable breeding material of rice resistant to rice blast, using the biotechnological approach for its further implementation in selection programmes and production.

Material and methods

The use of anther culture for increasing the homozygosity of the source material of the early generations of rice (Oryza sativa L.) hybrids is expedient due to a high level of heterozygosity of the selection offspring. This method significantly reduces the costs of human labour, material resources, and time for growing unpromising rice breeding material.

In the research, 20 rice genotypes were used as plant material: in 2017 – F, hybrids: M-202/Vikont (No. 207), Victoria/UIR-0548 (No. 210), Ukr NDS-6448/Victoria (No. 214), Brazos/Vikont (No. 209), and Labelle/Vikont (No. 221); in 2018 – F, hybrids: Labelle/UR-3470 (No. 221), Labelle/Delfino (No. 223), Brazos/Malysh (No. 225), Brazos/UR-3470 (No. 226), and Brazos/Delfino (No. 227); in 2019 – F, hybrid populations: Sirio/ Marshal (No. 207), Sirio/UR-4970 (No. 211), Labelle/Malysh (No. 236), Labelle/Chise Bind (No. 240), and Brazos/Malysh (No. 242); in 2020 – F, hybrid populations: Sirio/ Marshal (No. 245), Sirio/ Vikont (No. 246), Sirio/ Premium (No. 247), Sirio/ Debut (No. 248), and Sirio/UR-4970 (No. 249).

Parent forms of hybrids were carriers of genes for resistance to the causative agent of rice blast (Pyricularia oryzae): Pi-ta – Viscount, UIR-0548, Ukr NDS-6448, UIR-3470, Marshal, Premium, Debut, and UIR-4970 (country of origin – Ukraine), Chise Bind (Japan), and Delfino (Turkey). On the other hand, the carriers of the dominant Pi-b gene were M-202, Brasos, and Labelle (USA), Victoria (Russia), and Sirio (Italy). Identification of the donors of the relevant genes was carried out using DNA typing of plants (Galaev et al., 2015).

Donor plants of cultivated rice were grown in paddy fields at the experimental plots of the Rice Institute in Skadovsk district, Kherson Region, Ukraine. The panicles were cut when vacuolated microspores of most of the anthers were in the middle-late stage of the development. The cut panicles in the covering leaf were placed in water, wrapped with foil, and placed in a climate chamber at the temperature of +8–10°C for 4–5 days.

The cytological control of the development stage of microspores in anthers was conducted by preparing temporary micropreparations of anthers stained with acetocarmine under a light microscope. Panicles were freed from the covering leaves and placed in Petri dishes with a diameter of 150 mm.

Sterilisation was carried out as follows: panicles were poured over with a solution of the commercial product Bilyzna (including sodium hypochlorite (NaOCl), active chlorine, and alkalis) for 5 minutes and then drained. Further, the Petri dishes with panicles were filled with 0.05 N HCl (hydrogen chloride) solution for 10 minutes, after which they were washed five times with sterile distilled water.

Anthers were isolated aseptically in laminar box conditions and were cultured on the N6 nutrient medium in Petri dishes (φ = 60 mm) and exposed to the dark at +25°C for callus induction. The N6 nutrient medium was supplemented with 60 g L⁻¹ of sucrose and different phytohormones (Herath et al., 2010): NAA (α-naphthalene acetic acid) 1 mg L⁻¹, 2,4-D (2,4-dichlorophenoxyacetic acid) 3 mg L⁻¹, and kinetin 1 mg L⁻¹. After 3–6 weeks of cultivation, the callus was formed on the surface of the anthers. Subsequently, calli (1–2 mm in size) were transferred to the proliferation and regeneration Murashige and Skoog (MS) basal medium with addition of 0.75 mg L⁻¹ BAP (6-benzylaminopurine), 0.25 mg L⁻¹ kinetin, 0.25 mg L⁻¹ NAA, and 30 mg L⁻¹ maltose (Rukmini et al., 2013) with exposure to white light for 16 h at 28 ± 2°C until shoots development in about 2–4 weeks. Well grown shoots were transferred to the ½ MS basal medium for rooting. The percentage of calli and regeneration of green plants for each genotype was calculated from the number of planted anthers. The developed plantlets were acclimatised and transplanted into a greenhouse for further growth, seeding, and evaluation. The ploidy level of the regenerated doubled haploid (DH) plants was also evaluated by fertilisation analysis.

Field experiments of the phytopathological assessment of rice plants resistance to the causative agent of P. oryzae were conducted at the Rice Institute on an artificially created infectious background with controlled conditions: 27–28°C temperature and 98–100% air humidity (Figure 1).

The infectious background was created by treating the experimental plants with an aqueous suspension of spores of P. oryzae causative agent to 2–3 times a day, which were collected in the field in previous years and stored in a dry state. The experiment was repeated four times. As a control, rice standard cultivar ‘Vikont’ was used, as it is the most common in Ukraine. Determination of the degree of rice plants stability was carried out on a nine-point scale according to the generally accepted methodology (Table 1).

The evaluation of the obtained data and the correlation analysis of the level of resistance with the quantitative traits of rice were carried out by the methods of statistical analysis (Atramentova, Utjevskaya, 2007) with the software Excel.

Results

During 2016–2019, to combine the Pi-ta and Pi-b resistance genes in one genotype, 20 genotypes combinations of rice were created. At the next stage of the research, this hybrid material selected by the breeder was transferred to the Tissue Culture Laboratory for its stabilisation by the anther culture in vitro. The haploproduction capacity of microspores of five hybrid combinations provided by the breeder was evaluated annually, and the high responsibility of these forms to the provided in vitro conditions for the cultivation of anthers was shown (Table 2).
Figure 1. Infectious breeding ground: treatment of rice with a suspension of spores of the causative agent of *Pyricularia oryzae* (A), general appearance (B), and primary lesion of a rice leaf (C)

Table 1. Unified scale for evaluating the resistance of rice genotypes to *Pyricularia oryzae* according to plant damage (Dudchenko et al., 2015)

<table>
<thead>
<tr>
<th>Scale of damage</th>
<th>Resistance score</th>
<th>Degree of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1%</td>
<td>9–8</td>
<td>high resistant</td>
</tr>
<tr>
<td>2–5%</td>
<td>6–7</td>
<td>resistant</td>
</tr>
<tr>
<td>6–10%</td>
<td>5</td>
<td>medium resistant</td>
</tr>
<tr>
<td>11–25%</td>
<td>4</td>
<td>moderate susceptible</td>
</tr>
<tr>
<td>26–75%</td>
<td>3–2</td>
<td>susceptible</td>
</tr>
<tr>
<td>≥ 75%</td>
<td>0</td>
<td>high susceptible</td>
</tr>
</tbody>
</table>

Table 2. Efficiency of plant regeneration in other cultures of rice

<table>
<thead>
<tr>
<th>Year</th>
<th>Research material</th>
<th>Anther No.</th>
<th>Callus units</th>
<th>Callus %</th>
<th>Green regenerants units</th>
<th>Green regenerants %</th>
<th>Albino regenerants units</th>
<th>Albino regenerants %</th>
<th>LSD0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>F₁, M-202/Vikont</td>
<td>8461</td>
<td>287</td>
<td>3.39 ± 0.20</td>
<td>8</td>
<td>2.79 ± 1.03</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Victoria/UIR-0548</td>
<td>10592</td>
<td>366</td>
<td>3.46 ± 0.18</td>
<td>14</td>
<td>3.83 ± 1.00</td>
<td>3</td>
<td>0.82 ± 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Ukr NDS-6448/Victoria</td>
<td>10000</td>
<td>396</td>
<td>3.96 ± 0.20</td>
<td>13</td>
<td>3.28 ± 0.96</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Brazos/Vikont</td>
<td>12025</td>
<td>972</td>
<td>8.08 ± 0.25</td>
<td>113</td>
<td>11.63 ± 1.11</td>
<td>16</td>
<td>1.65 ± 0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Labelle/Vikont</td>
<td>9571</td>
<td>504</td>
<td>5.27 ± 0.23</td>
<td>5</td>
<td>0.99 ± 0.52</td>
<td>1</td>
<td>0.20 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>2018</td>
<td>F₁, Labelle/UIR-3470</td>
<td>4228</td>
<td>1656</td>
<td>39.17 ± 0.75</td>
<td>160</td>
<td>3.78 ± 0.29</td>
<td>24</td>
<td>0.57 ± 0.20</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>F₁, Labelle/Delfino</td>
<td>5625</td>
<td>1655</td>
<td>29.4 ± 0.61</td>
<td>66</td>
<td>1.16 ± 0.14</td>
<td>13</td>
<td>0.23 ± 0.06</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>F₁, Brazos/Malysh</td>
<td>5592</td>
<td>1981</td>
<td>35.43 ± 0.64</td>
<td>802</td>
<td>14.34 ± 0.47</td>
<td>61</td>
<td>1.09 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Brazos/UIR-3470</td>
<td>6338</td>
<td>1198</td>
<td>18.9 ± 0.49</td>
<td>166</td>
<td>2.62 ± 0.20</td>
<td>24</td>
<td>0.38 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Brazos/Delfino</td>
<td>4582</td>
<td>565</td>
<td>12.3 ± 0.49</td>
<td>75</td>
<td>1.64 ± 0.19</td>
<td>23</td>
<td>0.50 ± 0.10</td>
<td>0.60</td>
</tr>
<tr>
<td>2019</td>
<td>F₁, Sirio/Marshal</td>
<td>4892</td>
<td>1715</td>
<td>35.06 ± 0.68</td>
<td>120</td>
<td>2.43 ± 0.25</td>
<td>11</td>
<td>0.22 ± 0.02</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>F₁, Sirio/UIR-4970</td>
<td>6430</td>
<td>411</td>
<td>6.39 ± 0.30</td>
<td>20</td>
<td>0.31 ± 0.07</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>F₁, Labelle/Malysh</td>
<td>9329</td>
<td>2517</td>
<td>26.98 ± 0.46</td>
<td>280</td>
<td>3.0 ± 0.18</td>
<td>13</td>
<td>0.14 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Labelle/Chise Bind</td>
<td>4081</td>
<td>635</td>
<td>15.56 ± 0.57</td>
<td>59</td>
<td>1.45 ± 0.19</td>
<td>10</td>
<td>0.25 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Brazos/Malysh</td>
<td>6212</td>
<td>1264</td>
<td>20.35 ± 0.51</td>
<td>98</td>
<td>1.58 ± 0.16</td>
<td>27</td>
<td>0.40 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>2020</td>
<td>F₁, Sirio/Marshal</td>
<td>1965</td>
<td>1626</td>
<td>82.75 ± 0.85</td>
<td>144</td>
<td>7.33 ± 0.59</td>
<td>60</td>
<td>3.05 ± 0.39</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>F₁, Sirio/Vikont</td>
<td>3265</td>
<td>725</td>
<td>22.21 ± 0.73</td>
<td>48</td>
<td>1.47 ± 0.21</td>
<td>17</td>
<td>0.52 ± 0.13</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>F₁, Sirio/Premium</td>
<td>3299</td>
<td>1396</td>
<td>42.32 ± 0.86</td>
<td>56</td>
<td>1.70 ± 0.22</td>
<td>17</td>
<td>0.52 ± 0.12</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>F₁, Sirio/Debut</td>
<td>2369</td>
<td>638</td>
<td>26.93 ± 0.91</td>
<td>44</td>
<td>1.86 ± 0.28</td>
<td>8</td>
<td>0.34 ± 0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F₁, Sirio/UIR-4970</td>
<td>2813</td>
<td>1007</td>
<td>35.80 ± 0.90</td>
<td>85</td>
<td>3.02 ± 0.32</td>
<td>10</td>
<td>0.36 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>LSD0.05</td>
<td>2.96</td>
<td>1.20</td>
<td>0.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
As shown in Table 2, the level of callus formation in 2017 was low compared to other years of the research. Meanwhile, the regenerative capacity of the callus obtained was at the same level during all years of the research. Also, the callus formation level was observed for different rice lines. At the first stage of haploproduction in the culture of isolated anthers, the most productive was the $F_3$ hybrid Sirio/Marshal (No. 245) cultivated in 2020 with the callus formation level of $82.75 \pm 0.85\%$ of planted anthers. In 2017, the highest callus formation level was shown by the $F_2$ hybrids Brazos/Vikont (No. 219) and Labelle/Vikont (No. 221) with $8.08 \pm 0.25\%$ and $5.27 \pm 0.23\%$ of planted anthers, respectively. Three other hybrids cultivated in 2017 did not differ significantly in this indicator, and the level of callus formation was around 3.5%. In 2018, the highest callus productivity was shown by $F_2$ hybrids Labelle/UIR-3470 (No. 221) and Brazos/Malysh (No. 225) with $39.17 \pm 0.75\%$ and $35.43 \pm 0.64\%$, respectively. Finally, in 2019, $F_2$ hybrids Sirio/Marshal (No. 207) and Labelle/Malysh (No. 236) were characterised by the highest level of callus formation with the values of $35.06 \pm 0.68\%$ and $26.98 \pm 0.46\%$ of planted anthers, respectively.

In the previous studies (Shestopal et al., 2015), it was shown that changing the carbon source in the regenerative nutrient medium from sucrose to maltose increases the yield of green regenerating plants. The results of this study showed that the regeneration ability of the obtained callus was different. However, it should be noted that green regenerants were obtained from all 20 rice genotypes, and the number of albino plants was low (Table 2).

The regenerated green plants formed on the medium for regeneration (Figure 2A) were transplanted to a hormone-free MS basal medium with half the concentration of salts (Figure 2B), and then plants with a well-developed root system were planted in the soil in a mini-greenhouse (Figure 2C) for the adaptation to ex vitro conditions.

In the biotechnology of obtaining the linear material by androgenesis in vitro, the stage of adaptation (Figures 2c and 3) is one of the most important due to a high percentage of plants dying at this stage for various reasons: severe water deficit in tissues after transfer from in vitro conditions, chromosomal imbalance of regenerants, etc. (Tripathy, 2018).

In the current research, a set of conditions for the adaptation of regenerating plants of sowing rice was implemented, which resulted in a high green plants survival rate – 93.9% in 2016, 62.8% in 2018, and 62.3% in 2019. In 2019, detailed research of the process of adaptation of regenerated plants to ex vitro conditions as the most vulnerable stage of any in vitro biotechnology was conducted. It was shown that during adaptation in a nutrient medium and in the soil, on average, 39% of regenerants obtained in the culture die (Figure 4).

At the next stage of growing of regenerants, up to 20% of already adapted plants die due to various reasons such as chromosomal (genomic) instability, diseases, etc. Thus, approximately $\frac{1}{3}$ (34.3%) of rice regenerants obtained by androgenesis in vitro will give a seed generation, and this fact must be considered by researchers at the stage of planning an experiment.

Next, in 2017–2020, the testing of the morphogenetic potential of 15 $F_2$ hybrids and five $F_3$ ones in the anther culture of seed rice was conducted. Using the technology of androgenesis in vitro, 1057 regenerated plants were obtained. This research was performed in close cooperation with an employee of the Rice Institute.
Figure 4. Dynamics of the number of regenerated rice plants at the stages of adaptation and growing (as a percentage of the obtained regenerants)

Table 3. Resistance of rice genotypes and lines to *Pyricularia oryzae* under conditions of infectious breeding ground (2020)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cross combination</th>
<th>Resistance type of the lines</th>
<th>resistant</th>
<th>medium resistant</th>
<th>susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>‘Vikont’ (standard)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>225/1</td>
<td>225/2, 225/3, 225/4, 225/6</td>
<td>225/5</td>
</tr>
<tr>
<td>UIR-9743/K-01474</td>
<td>Labelle/Malysh</td>
<td>236/2, 236/9, 236/11, 236/12, 236/16</td>
<td>236/13, 236/14</td>
<td>236/4, 236/6, 236/7</td>
<td>236/8, 236/10, 236/15, 236/17, 236/18, 236/19, 236/20</td>
</tr>
<tr>
<td></td>
<td>Labelle/UIR-3470</td>
<td>240/3</td>
<td>240/1, 240/2</td>
<td>242/1</td>
<td>242/2, 242/3</td>
</tr>
<tr>
<td></td>
<td>Brazos/Malysh</td>
<td>242/1</td>
<td>207/5, 207/7, 207/8</td>
<td>207/1, 207/2, 207/3, 207/4, 207/6, 207/9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sirio/Marshal</td>
<td>211/2</td>
<td>211/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sirio/UIR-4970</td>
<td>211/2</td>
<td>211/1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

According to Table 3, in the growing season of 2020, five lines of the combination Label/Malysh and one of each of the combinations UIR-9743/K-01474, Label/UIR-3470, and Brazos/Malysh with a resistance index of 7–9 were resistant to rice damage. The intermediate level of resistance was determined in the following lines: ‘Vicont’ (standard), five of Label/Malysh, four of UIR-9743/K-01474, tree of Sirio/Marshal, two of each Label/UIR-3470 and Brazos/Malysh, and one of Sirio/UIR-4970. Other lines were susceptible to rice blast.

Correlation of resistance to the causative agent of rice blight with other quantitative traits in regenerating rice lines was determined (Figure 5).

According to the obtained data, a significant positive correlation ($r = 0.257 \ldots 0.685$) was found between resistance to rice blast and the number of spikelets and grains in the panicle, its productivity, and the whole plant productivity. It proves that severe damage to plants leads to a significantly high level of emptiness of the panicle: in this case, a significant level of the negative correlation ($r = -0.320 \ldots -0.278$) was found. This tendency was observed for foliar, nodular, and panicle forms of the disease.

According to the obtained data (Shpak, 2015), almost all studied lines (except 236/2 Labelle/Malysh) were characterised by short stems compared to the standard cultivar as well as a high level of bushiness (7–26 shoots compared to 4 of the standard). Moreover, the lines 225/1 UIR-9743/K-01474, 240/3 Labelle/UIR-3470, and 242/1 Brazos/Malysh had the highest length of the main panicle (21.1–24.2 cm compared to 19.5 cm of the standard). The productivity of the studied forms of rice in the conditions of severe damage by the causative agent of rice blast was determined. The highest weight of
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1000 grains was shown by the line 236/9 Labelle/Malysh, which significantly exceeded the standard cultivar (32.53 g compared to 30.95 g). The productivity of the main panicle was high in the following lines: 225/1 UIR-9743/K-01474, 236/2 Labelle/Malysh, 236/9 Labelle/Malysh, and 240/3 Labelle/UIR-3470 (5.32–8.69 g compared to 4.55 g of the standard).

**Discussion**

To accelerate the selection programmes for many crops including rice, the anther culture method has been successfully used (Tripathy, 2018; Samantaray et al., 2021). The question of which rice genetic material can be used as a donor for the androgenesis *in vitro* is controversial. The use of F₂ hybrids increases the duration of selection by one year. Nevertheless, there are several arguments in favour of F₂ hybrids. In some years, the F₁ hybrid offspring do not respond to the conditions of anther culture *in vitro*: in rice, only hybrids of F₂ generation can be used for the anther culture. The frequency of callus formation is higher in F₂ hybrids than in F₁ ones (Bishnoi et al., 2012).

The success of androgenesis is determined by such factors as the genotype, physiological state of the donor plant, stage of development of microspores in the anthers taken into culture, composition, and physical state of the nutrient medium, cultivation conditions, and preliminary treatment of anthers (Lal Dalpat et al., 2014; Ruwani et al., 2018; Tripathy et al., 2019b). The anther cultivation technique has been shown to be efficient for the breeding of Japonica rice, while its application to Indica rice remains limited mainly due to their relatively weak androgenic response with some exceptions (Naik et al., 2017).

The results of the research showed that applying the methodology for rice androgenesis *in vitro* developed in the laboratory ensured the production of green regenerants for all 20 rice genotypes of the Indica subspecies involved in the process. It should be emphasized that a significant level of callus formations was shown for all given hybrid combinations. Kaushal et al. (2014) reported that among seven Indica rice cultivars on anther culture, callus formation rates varied from 17.24% to 40.64%, while the green plant regeneration efficiency ranged from 11.43% to 40.93%. Similar results were obtained in the current research. Thus, callus formation ranged between 3.39% and 82.75% of planted anthers, and the regeneration of green plants level varied from 0.16% to 13.34%.

An interesting feature of the passage of the first stage of the androgenesis *in vitro* in the rice anther cultures (e.g., as opposed to wheat) is the undulation of callus formation. The first calli appear on the surface of anthers on the 4–5th day of cultivation, and they are transplanted to the regenerative nutrient medium. Next, after 7–9 weeks of cultivation, the second wave of callus appeared. According to the visual observations, the intensity of callus formation was higher at the first stage, and the callus that appeared on the surface of anthers first had the maximum ability to regenerate green plants. Moreover, the longer the time from the planting of anthers to callus appearing, the lower the ability of the latter to regenerate plants. Therefore, the haploproduction capacity of the callus is inversely proportional to the speed of its formation.

One of the limiting factors that reduce the effectiveness of the biotechnology of creating rice doubled haploids is a large number of albino plants (Tripathy, 2021). Detailed studies of proplastids and plastid genomes of regenerated albino plants have shown that albinism mainly appears due to an incomplete formation of membrane structures and various blockages in plastid development, namely, large-scale deletions and rearrangements in plastid genome. In literary sources, there are reports of almost equal number of plants with normally developed chloroplasts and chlorophyll-defective plants. Thus, in a study of Mishra et al. (2015), the percentage of green regenerants obtained was 10.28–37.23% and albinoplants constituted 12.38–17.11%. In the current research, the percentage of green regenerants was significantly higher than the level of albino plants production: the average of 3.31% **versus** 0.51%.

**Figure 5.** Correlation of the resistance to the causative agent of *Pyricularia oryzae* with quantitative traits in rice (infectious breeding ground, 2020)
Consequently, it is possible to state that the developed technology is efficient for obtaining the linear material of seed rice for the specific genetic material; however, it still requires additional experiments involving donor material from another region (or breeding centre).

Adaptation of regenerants to ex vitro conditions and their further growing are among the most important stages of the biotechnology of obtaining rice lines in androgenesis in vitro. The percentage of acclimatised plants according to various publications ranges from 60% to 80% (Ilyushko, Romashova, 2019; Pattnaik et al., 2020; Shestopal et al., 2020). According to the results of the current research, at the stage of adaptation to the soil, survived from 45% to 70% of green regenerants. However, not all these plants had the genetic determinants that allowed the plant to undergo further stages of growth differentiation and seeds production. Normally, plants obtained in anther culture can be considered haploids because they have arisen from haploid microspores. However, the actual plants produced during regeneration might be a mixture of haploids, diploids, or mixoploids, which can be explained by the defects in the development of microspores or callus tissue (Ilyushko, Romashova, 2019). The fusion or unequal division of nuclei, endomitosis inside the pollen grain, and disruption of meiosis could also lead to the development of plants other than haploids.

Rice is characterised by a spontaneous doubling of chromosomes in the haploid cells of the callus, which leads to the formation of numerous doubled haploids. The rate of the spontaneous doubling of chromosomes depends on the type of anther culture and cultivation conditions and varies from 30% to 87% (Mishra, Rao, 2016). The proportion of doubled haploids and other fractions of regenerants varies depending on rice subspecies, callus cultivation conditions, method of obtaining regenerants and other factors. When growing in greenhouses of the Rice Institute, seed offspring were obtained from 35% of transferred acclimatised plants, on average. In the current research, diploides were not used suggesting that all fertile plants were the result of spontaneous diploisation.

Next, the obtained rice lines were evaluated in artificial and field conditions by specialists conducting selection for resistance to rice blast. It should be noted that in the infectious breeding ground, the conditions contributing to the intensive development of the pathogen were created. Consequently, a rather high proportion of studied lines was attributed to susceptible forms. Only eight lines appeared to be resistant and showed 9 points of resistance, while 18 lines had an intermediate level of resistance to the disease.

A number of parameters describing the habit and productivity of obtained stable rice lines were also investigated. It was shown that most of them were characterised by a high level of plant productivity (21.80–60.42 g in comparison to 10.31 g of the standard), which can be explained by a high productive bushiness. In particular, the lines 236/2 Labelle/Malysh and 236/9 Labelle/Malysh showed, on average, higher values in the most of the studied productivity indicators compared to the standard cultivar ‘Vikont’.

Thus, the effectiveness and perspective of the creation of rice lines resistant to rice blast by androgenesis in vitro was revealed. However, the developed techniques still do not guarantee obtaining a sufficient number of gene plasma lines of various origins for research. Nevertheless, the most important tool for breeders has become the anther culture in vitro, the use of which allows not only reduce the time of creating rice cultivars, but also fix valuable recombinations.

Conclusions

1. By the method of anther culture in vitro, 1,057 regenerated rice plants were obtained and transferred to the Rice Institute in Skadovsk, Ukraine for further cultivation and research.

2. The most responsible to the cultivation conditions was the F1 hybrid Sirio/Marshal (No. 245): the percentage of calli from planted anthers was 82.75 ± 0.85, and the percentage of green plants’ regeneration was 7.33 ± 0.59.

3. It was found that only 34.3% of regenerated rice plants obtained by androgenesis in vitro produced seed generation, which must be considered by researchers at the stage of experiment planning.

4. The rice blast resistance of rice lines was studied by the anther culture technology. It was found that a high level of resistance (9 points) showed lines 225/1 UIR-9743/K-01474, 236/2, 236/9, 236/11, 236/12, 236/16 Labelle/Malysh, 240/3 Labelle/UIR-3470, and 242/1 Brazos/Malysh.

5. A significant positive correlation (r = 0.257 ... 0.685) was found between the level of resistance to rice blast and the number of spikelets and grains in the panicle, its productivity, and the productivity of the plant.

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Stabilisation of promising breeding rice plants resistant to Pyricularia oryzae


