**Image J – leaf litter scan protocol (Using ImageJ/FIJI 1.46r)**

1. Leaf litter scans 10mm x 10mm squares with single leaf litter within.
2. Crop individual pictures from pdf, and save as highest quality jpeg. The saved name should be the litter type (green/rooibos)+**scan page**+square number, e.g. Green**1a**1.
3. Open images in Image J. Drag and drop all 110 green/rooibos leaf litter images simultaneously into image J. This allows you to open multiple images at the same time rather than individually into image J.
4. Create a stack. Follow the tabs Image > Stacks > Images to stack and the then save the stack name as range of scans e.g. Green1a1-110 (scans 1 to 110). The method should be copy to center and ensure the use of title as labels is ticked. If the some of the cropped images are larger it creates black border along smaller images. Use Flood fill tool to fill the borders white (i.e remove the black borders) Otherwise, this will be wrongly treated as part of the leaf analysis later.
5. Split colour channels. Image> Color> split channels. We are only analyzing the blue channel as this has the greatest contrast between leaf and white background – delete other channels.
6. Standard threshold for holes. Adjust>Threshold (Default/Red)🡪 SET to 170-255 (top moveable bar 170 and bottom set at 255). Do not tick dark background. A new window will pop up to create binary image – uncheck dark background and uncheck adjust threshold for each image – they need the same threshold. Close threshold window. The image will now be black with holes as white.
7. Customize summary output. Analyze > Set Measurements > Area, Area fraction and Limit to threshold. Only tick Area, Area fraction and Limit to threshold – all others are unchecked.
8. Analyse>Particle analysis>summarise> Clear results, summarize and in situ show are only ticked.
9. Save summary table. This will save the summary table as a text file. Repeat for the 110 rooibos leaves.
10. To measure the number of pixels in the 10x10mm square. Crop one empty square and load it in ImageJ. Choose the Straight line tool by clicking it, and draw a line across one side of the square while pressing the “shift” button to keep it straight.
11. Find the scale. Analyse>Set Scale. In “known distance”-box write “10”, and in “unit of length”-box write “mm”. Write down the scale (i.e 12.5333pixels/mm= length of the square. The area is then 157pixels2/mm2 )
12. In excel, calculate the area (mm^2) of a leaf by using values from ImageJ analysis: Total area (the area in pixel^2 that is the leaf) and the area of the squares. Use the following formula: